Characterization of Carbohydrates in Oyster (Crassostrea virginica) Hemolymph

Robert Joel Lowy

College of William and Mary - Virginia Institute of Marine Science

Follow this and additional works at: https://scholarworks.wm.edu/etd

Part of the Biochemistry Commons

Recommended Citation

https://dx.doi.org/doi:10.25773/v5-9v6d-k328
CHARACTERIZATION OF CARBOHYDRATES IN OYSTER (CRASSOSTREA VIRGINICA) HEMOLYMPH

A Thesis
Presented to
The faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Master of Arts

by

R. Joel Lowy
1977
APPROVAL SHEET

This thesis is submitted in partial fulfillment of
the requirements for the degree of
Master of Arts

Robert Joel Lowy

Approved, August, 1977

Dexter S. Haven, M.S.

Frank O. Perkins, Ph.D.

Bruce J. Neillson, Ph.D.

J. Ernest Warriner, III, M.A.

Richard L. Wetzel, Ph.D.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>2</td>
</tr>
<tr>
<td>METHODS AND MATERIALS</td>
<td>4</td>
</tr>
<tr>
<td>SOURCE OF OYSTERS</td>
<td>4</td>
</tr>
<tr>
<td>COLLECTION OF HEMOLYMPH SAMPLES</td>
<td>5</td>
</tr>
<tr>
<td>COLORIMETRIC ANALYSIS</td>
<td>8</td>
</tr>
<tr>
<td>HEMOLYMPH EXTRACTION AND FRACTIONATION PROCEDURES</td>
<td>12</td>
</tr>
<tr>
<td>THIN LAYER CHROMATOGRAPHY</td>
<td>17</td>
</tr>
<tr>
<td>COLORIMETRIC RESULTS</td>
<td></td>
</tr>
<tr>
<td>EVALUATION OF EXTRACTION TECHNIQUES FOR HEMOLYMPH</td>
<td></td>
</tr>
<tr>
<td>CARBOHYDRATES</td>
<td>22</td>
</tr>
<tr>
<td>COMPARISON OF TOTAL APS, FCHO AND PCHO IN HEMOLYMPH</td>
<td>25</td>
</tr>
<tr>
<td>FROM FIELD SAMPLES</td>
<td></td>
</tr>
<tr>
<td>SEASONAL VARIATION IN TOTAL APS, FCHO, PCHO AND A-B CHO IN FIELD AND</td>
<td>31</td>
</tr>
<tr>
<td>LABORATORY OYSTERS</td>
<td></td>
</tr>
<tr>
<td>SPECIFIC ENZYMATIC COLORIMETRIC METHODS FOR GLUCOSE AND Trehalose</td>
<td>36</td>
</tr>
<tr>
<td>SUMMARY OF CARBOHYDRATE COMPOSITION OF OYSTERS BASED ON COLORIMETRIC</td>
<td>47</td>
</tr>
<tr>
<td>FACTS</td>
<td></td>
</tr>
<tr>
<td>TABLE OF CONTENTS (CONTINUED)</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>THIN LAYER CHROMATOGRAPHY RESULTS</strong> .............................................. 49</td>
<td></td>
</tr>
<tr>
<td><strong>EVALUATION OF SEVERAL EXTRACTION AND DESALTING TECHNIQUES</strong> ................... 49</td>
<td></td>
</tr>
<tr>
<td><strong>IDENTIFICATION OF OYSTER HEMOLYMPH FREE CARBOHYDRATE</strong> ....................... 52</td>
<td></td>
</tr>
<tr>
<td><strong>DISCUSSION</strong> ................................................................. 72</td>
<td></td>
</tr>
<tr>
<td><strong>ANALYTICAL TECHNIQUES</strong> ........................................................ 72</td>
<td></td>
</tr>
<tr>
<td><strong>COMPOSITION OF OYSTER HEMOLYMPH</strong> ............................................... 74</td>
<td></td>
</tr>
<tr>
<td><strong>GEOGRAPHIC AND SEASONAL VARIATION</strong> ............................................. 79</td>
<td></td>
</tr>
<tr>
<td><strong>SUMMARY</strong> ................................................................. 87</td>
<td></td>
</tr>
<tr>
<td><strong>APPENDIX A - ANTHRONE REACTION</strong> ............................................... 88</td>
<td></td>
</tr>
<tr>
<td><strong>APPENDIX B - HEXOSAMINE REACTION</strong> ............................................. 95</td>
<td></td>
</tr>
<tr>
<td><strong>APPENDIX C - GLUCOSE OXIDASE DETERMINATION OF GLUCOSE</strong> ................. 101</td>
<td></td>
</tr>
<tr>
<td><strong>APPENDIX D - FLUOROMETRIC DETERMINATION OF GLUCOSE</strong> ......................... 106</td>
<td></td>
</tr>
<tr>
<td><strong>APPENDIX E - ENZYMATIC Trehalose Determination</strong> ........................... 112</td>
<td></td>
</tr>
<tr>
<td><strong>APPENDIX F - TLC VISUALIZATION METHODS</strong> ...................................... 115</td>
<td></td>
</tr>
<tr>
<td><strong>BIBLIOGRAPHY</strong> .............................................................. 120</td>
<td></td>
</tr>
<tr>
<td><strong>VITA</strong> ................................................................. 125</td>
<td></td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

The author expresses his appreciation to the members of the committee for their helpful suggestions and critical reading of the manuscript. Particular thanks is due to Mr. Dexter Haven for his support and encouragement throughout the program. Special gratitude is extended to Mr. Haven, Dr. Perkins, Mr. Warinner and Dr. Zubfoff, Dr. Wetzel, and the members of their departments, as well as the Aquaculture Department, for use of laboratory equipment and facilities, and their help and courtesy. Dr. Paul Zubkoff and Nancy Windsor also provided many fruitful discussions and helpful suggestions. Additional thanks is extended to Teresa Wilburn for her typing skills and the VIMS Art Department, particularly Junè Hoagman.

Finally, I extend my appreciation and gratitude to my parents and my Lord Jesus Christ, without whose loving support and encouragement this thesis would not have been possible.
LIST OF TABLES

Table                                                                 Page
1. Hemolymph Sampling Conditions .............................................6
2. Comparison of Carbohydrate Extraction Methods .......................23
3. Field Survey Data ................................................................30
4. Summary of Colorimetric Data for Hemolymph Carbohydrates .........35
5. Glucose Oxidase Determination of Hemolymph Glucose .................38
6. Hexokinase-NADPH Determination of Hemolymph Glucose .............42
7. Enzymatic Assay of Trehalose ..................................................45
8. Thin Layer Chromatography Mobility Data ...............................68
9. Comparative Hemolymph Carbohydrate Data ...............................84
10. Anthrone Standard Curves .......................................................91
11. Acid-Base Hydrolysis and Anthrone Determination of Representative Carbohydrates ..........................92
12. Hexosamine Standard Curve ...................................................98
13. Glucose Oxidase Standard Curve .............................................103
15. Trehalose Assay Reaction Mixtures .........................................114
16. TLC Visualization Reagent Results .........................................118
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Oyster hemolymph carbohydrate assays</td>
<td>9</td>
</tr>
<tr>
<td>2. Fractionation and extraction procedures</td>
<td>13</td>
</tr>
<tr>
<td>3. Total APS versus wet weight</td>
<td>24</td>
</tr>
<tr>
<td>4. Total APS versus dry weight</td>
<td>29</td>
</tr>
<tr>
<td>5. Time variation in hemolymph and environmental factors</td>
<td>34</td>
</tr>
<tr>
<td>6. Effect of salinity on glucose and trehalose mobility</td>
<td>57</td>
</tr>
<tr>
<td>7. Comparison of extraction - desalting methods for TLC</td>
<td>59</td>
</tr>
<tr>
<td>8. Comparison and preliminary identification of carbohydrates in three hemolymph samples</td>
<td>61</td>
</tr>
<tr>
<td>9. TLC identification of hemolymph carbohydrates II</td>
<td>63</td>
</tr>
<tr>
<td>10. Identification of hemolymph carbohydrates II</td>
<td>65</td>
</tr>
<tr>
<td>11. Identification of carbohydrates by differential visualization methods</td>
<td>67</td>
</tr>
<tr>
<td>12. TLC mobilities versus hexose unit number</td>
<td>71</td>
</tr>
<tr>
<td>13. Anthrone standard curve</td>
<td>94</td>
</tr>
<tr>
<td>14. Hexosamine standard curve</td>
<td>100</td>
</tr>
<tr>
<td>15. Glucose oxidase standard curve</td>
<td>105</td>
</tr>
<tr>
<td>16. HK-NADPH glucose determination standard curve</td>
<td>110</td>
</tr>
</tbody>
</table>
ABSTRACT

Colorimetry and thin layer chromatography techniques have been developed and used to characterize oyster hemolymph carbohydrate (CHO). The non-specific anthrone determination has been coupled with TCA-ETOH fractionation procedure to measure total anthrone positive substances (total APS), TCA-ETOH soluble substances (FCHO) and TCA-ETOH insoluble substances (PCHO). The FCHO fraction was further treated by an acid-base technique to obtain highly chemically stable carbohydrates (A-B CHO). The total APS changed from 1100 µg/ml in the winter, falling to 234 µg/ml by April and returned to 1200 µg/ml by May. Most of this change was due to fluctuations in the PCHO. As a proportion of the total APS, PCHO was least constant and FCHO most constant. The hemolymph CHO change seemed too early to correlate with gametogenesis, but did follow condition index. It was hypothesised that, the change is nutritionally caused, due to an increase in metabolic activity above assimilation. Field samples taken from VIMS (York River), the Rappahannock River and Eastern Shore showed little difference in any fraction regardless of geographic origin, animal size, or salinity. Although there is a great deal of variation in total APS between individual oysters, total APS or other hemolymph fractions could be used as indicators of nutritional state in field and laboratory experiments.

The glucose oxidase and a fluorometric modification of hexokinase-NADPH enzymatic glucose determinations were tried. Oyster hemolymph was found to have approximately 40 µg/ml of glucose, which is below or near the detection limits of these methods. An enzymatic determination of trehalose, using trehalase, was not completely successful, but did indicate that levels are quite low. Glucose was concluded to be the primary "blood sugar". A large proportion of the PCHO and A-B CHO remained unidentified. It is hypothesised a large amount of these fractions may be maltotriose or other oligosaccharides, but the physiological function is unknown.

The TLC data showed the following carbohydrates to be present, in order of concentration: maltotriose, glucose, maltose/trehalose, and galactose.

In concentration and composition oyster hemolymph is quite similar to that determined for other mollusca and the crustaceans.
CHARACTERIZATION OF CARBOHYDRATES
IN OYSTER (CRASSOSTREA VIRGINICA)
HEMOLYMPH
INTRODUCTION

Despite considerable information about the carbohydrate metabolism of the mollusca, relatively little is known about the carbohydrates of the hemolymph and which ones are metabolically active as blood sugar (Goudsmit, 1972). Most values, with the exception of the gastropods, are relatively old and done with procedures which measure total reducing carbohydrates rather than glucose or even free monosaccharides specifically (Martin, 1961; Goddard and Martin, 1966). Information on the lamellibranchs is particularly sparse. Therefore, an attempt to characterize the free carbohydrates and identify the blood sugar of the oyster is a useful undertaking.

Glucose was considered the most likely major constituent of the hemolymph and blood sugar, with trehalose a secondary possibility. Glucose is of widespread importance, including in the gastropods (Marques and Falkner, 1976).

Trehalose, until recently, was considered the predominate insect blood sugar (Florkin and Jeuniaux, 1964; Bedford, 1973; Friedman, 1970). It occurs in a great number of invertebrates, including various molluscs and the oyster (Fairbain, 1958; Badman, 1967). Trehalose, while not predominant, occurs in the hemolymph of several marine crustaceans (Telford, 1965, 1968b,c). It seems to have a
special physiological function in the brine shrimp and crayfish (Boulton and Huggins, 1977; Schwoch, 1972. Badman (1967) found the trehalose content of *Crassostrea virginica* to be relatively low, but it did vary seasonally with fluctuations in glycogen (Badman, 1967). He suggested that, unless it was restricted to a specific tissue such as the hemolymph, it was unlikely that trehalose had a significant storage role. He also speculated it might be important in aiding glucose adsorption in the gut, as found in locust (Treherne, 1958a).

In light of this background several questions were considered:

1. What carbohydrates are present in oyster hemolymph; specifically are glucose and/or trehalose present?

2. What carbohydrate(s) seem to predominate and be regulated, suggesting the role of blood sugar?

3. If trehalose is present, is it in high enough concentration to suggest an important storage role?

An additional goal of this study was to investigate various carbohydrate measurement techniques and find those applicable for lamellibranchs. Since the composition of the arthropods's hemolymph have been much more thoroughly investigated, these studies were used as a basis to develop the methods for the present project (Telford, 1965, 1968b, c; McWinnie and Saller, 1960; Wyatt and Kalf, 1957).
METHODS AND MATERIALS

Two analytical approaches have been used to characterize oyster hemolymph. The first was to use specific and nonspecific carbohydrate colorimetric tests combined with chemical fractionation. This allowed quantitative measurement of broad categories of carbohydrates. The hemolymph, both from laboratory populations of oysters held at VIMS, as well as a few field populations, has been characterized in this manner. The second method was to use thin layer chromatography (TLC) for qualitative identification of the free carbohydrates present.

Source of Oysters

The laboratory populations of oysters were held at VIMS in flumes of free flowing York River water, and no attempt was made to control environmental parameters. Flumes were drained and feces and pseudofeces washed away daily, with a thorough cleaning once a week. Animals were held at least two weeks at VIMS before using the hemolymph for any study. Most of the animals were originally collected from the Rappahannock River but some were from the Great Wicomico River. These regions were chosen as being relatively free of disease and pollution. Animals were sampled at various intervals, as needed for biochemical
characterization (see Table 1 for location and dates of sampling and hydrographic conditions). It is noted that the number of animals used, the time intervals and the hemolymph pooling was not constant. All animals used were between 8 and 13 centimeters long and randomly chosen from the flume.

For the field survey fifty animals were taken from the Eastern Shore at Wachapreague, the Rappahannock River at Smokey Point and from the flumes at VIMS. The latter group were originally from the Rappahannock, but had been held at VIMS for five weeks. The three groups averaged from 8 to 13 centimeters and were typical of the mature oysters of the areas. Hemolymph from twenty five animals from each area was taken in the field and stored in individual vacutainers (Becton, Dickinson and Company, Rutherford, New Jersey). In addition, one ml of hemolymph from each of the 25 remaining animals was pooled. Samples were transported on ice to VIMS and immediately frozen and stored at -20°C until analysis.

Collection of Hemolymph Samples

Hemolymph was taken from the adductor sinus in the following manner (Dr. F. O. Perkins, personal communication). For the biochemical characterization on animals held at VIMS, animals were chilled in a refrigerator for 30 minutes before sampling. This minimized the animals' response to handling and therefore the possible changes in hemolymph carbohydrate composition and concentration. In the field study animals could not be chilled. Therefore those used
### TABLE 1

**Hemolymph Sampling Conditions**

<table>
<thead>
<tr>
<th>Hemolymph Sample Date 1977</th>
<th>Origin of Oysters Date to VIMS</th>
<th>Field Values Temp. °C. Salinity</th>
<th>Laboratory Values Temp. °C. Salinity</th>
<th>Number of Oysters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan 23</td>
<td>Rapph. R. Fall '76</td>
<td>0</td>
<td>22</td>
<td>4 Individuals</td>
</tr>
<tr>
<td>March 1</td>
<td>Grt. Wicom. Feb. 25</td>
<td>6.5</td>
<td>22</td>
<td>3 Pooled</td>
</tr>
<tr>
<td>March 17</td>
<td>Rapph. R. March 9</td>
<td>12.0</td>
<td>22</td>
<td>10 Pooled</td>
</tr>
<tr>
<td>April 1</td>
<td>Rapph. R. March 9</td>
<td>14.0</td>
<td>22</td>
<td>4 Individuals</td>
</tr>
<tr>
<td>May 2</td>
<td>Rapph. R. April 7</td>
<td>18.0</td>
<td>20</td>
<td>10 Pooled</td>
</tr>
<tr>
<td>May 16 (E. Shore)</td>
<td>E. Shore</td>
<td>22</td>
<td>28</td>
<td>25 Pooled</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25 Individuals</td>
</tr>
<tr>
<td>May 18 (VIMS)</td>
<td>Rapph. R. April 7</td>
<td>21</td>
<td>18</td>
<td>&quot;</td>
</tr>
<tr>
<td>May 31 (Rapph. R.)</td>
<td>Rapph. R.</td>
<td>23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
from VIMS for the field study were not refrigerated either prior to taking hemolymph samples. The shells were cleaned to remove mud and epiphytes. Next, an oyster knife was inserted into the hinge and the hinge ligament broken. The knife was then used to pry a small gap so that the location of the adductor could be observed. As little strain as possible was placed on the adductor so as to prevent tearing the muscle, which would result in loss of hemolymph and contamination of the sinus with shell liquor. The animal was then tipped to one side to allow shell liquor to drain away from the adductor. A 1 ml tuberculin disposable syringe with a 22.gauge needle was inserted into the muscle to withdraw the hemolymph. Care was taken to keep the needle tip within the muscle tissue.

No attempt was made to obtain cell-free extracts. Hemocytes rupture easily and it would be difficult to prove that cell rupture had not occurred, either in drawing the hemolymph or during centrifugation or any other separation process. Samples were always frozen after collection and before analysis to assure total cellular disruption. Therefore, values presented in this thesis are for the hemolymph, but do not differentiate between the intracellular and extracellular compartments. All samples were stored at -20°C until analysis. Generally samples were analyzed within a few days, but some analyses were done as much as a month later.
Colorimetric Analysis

A variety of colorimetric methods was used to characterize the hemolymph, the anthrone reaction in combination with chemical fraction being used most extensively. A summary of the various tests made on hemolymph appears in Figure 1; details of the methods and representative data appear in the Appendices. A brief description of the substances measured by each method and the fractionation procedures used follow:

I. **Total Anthrone Positive Substitutes** (Total APS)

The anthrone reagent is used directly on the sample with glucose as the standard. Since anthrone is non-specific, it will include all mono-, oligo-, and polysaccharides, glycoproteins, and agglutination factors present in the hemolymph, both free and as part of cellular components (Appendix A).

II. **TCA-ETOH Soluble Material** - Free Carbohydrates (FCHO)

Anthrone reagent was used on TCA-ETOH (tricloroacetic acid - ethyl alcohol) supernatant with glucose as the standard. All small molecules including monosaccharides, disaccharides and most low molecular weight oligosaccharides are in this fraction. Although TCA treatment removes proteins, some low molecular weight glycoproteins may be in this fraction (Figure 2 B, Appendix A).

III. **TCA-ETOH Insoluble Material** - Polysaccharides (PCHO)

After precipitation with the ETOH the PCHO material was taken up in distilled water and anthrone reagent was used on an aliquot of this solution. One would expect most of this
material to be glycogen. However, it does include any other high molecular weight carbohydrates polymers that might be present such as chitin, galactogen and mucopolysaccharides. It also may include some oligosaccarides and glycoproteins due to co-precipitation with the polysaccharide material (Figure 2 B, Appendix A).  

IV. Acid-Base Non-Labile Material (A-B FCHO)

This procedure is based on a method for quantative determination of trehalose in insect hemolymph (Wyatt and Kalf, 1957). An aliquot of the FCHO fraction containing 20 to 100 µg is heated 10 minutes in a boiling water bath, first with 0.1 N HCl and then with 6 N NaOH. This procedure was found to hydrolyze most mono- and disaccharides essentially to completion. The anthrone reagent is added to the reaction mixture directly. A regular glucose standard and an acid-base-treated trehalose standard are used. Appendix A presents data, confirming Wyatt and Kalf, (1957), that relatively little of any carbohydrate except trehalose remains after this process. Chitin, chitin precursors, and protected glycoproteins may also be non-labile, but were not tested (Dr. P. Zubkoff, personal communication) (Figure 2 C, Appendix A).  

V. Glucose - Glucose Oxidase Method

An enzymatic clinical diagnosis kit for determination of (human) blood glucose from Sigma Chemical Co. was used. Procedures followed were as recommended by Sigma Technical Bulletin No. 510. Frozen and fresh hemolymph, both deproteinized and non-deproteinized, were used for this test. The test is based on the coupled enzymatic
reactions of glucose to gluconic acid, via glucose oxidase. The resulting peroxide from this reaction oxidizes a chromophor, O-dianisdine, via peroxidase, forming a colored species (Appendix C).

VI. Glucose - Hexokinase Method

This is another enzymatic clinical diagnostic procedure from Sigma Chemical Co. (St. Louis, Mo., Technical Bulletin No. 15-UV). The test is based on the conversion of glucose to glucose-6-phosphate catalyzed by hexokinase. Then glucose-6-phosphate dehydrogenase converts the glucose-6-phosphate to 6-phosphogluconic acid, with the reduction of NADP (nicotinamide adenine dinucleotide phosphate) to NADPH. Usually the change of absorbance due to NADPH is measured; however, a fluorometric modification (Quilter, 1977 after Scherstein and Tibbling, 1967) increases the sensitivity greatly and was used in this study (Appendix D).

VII. Trehalose - Enzymatic Method

Experiments to measure trehalose directly and specifically by coupled enzymatic tests were done using a method based on modifications of Hey and Elbein (1968) and Rutherford and Jefferson (1976). The technique involves incubation of the sample with enzyme trehalase (obtained from Dr. A. D. Elbein) which hydrolyzes trehalose into its two glucose units. The glucose can then be measured by any colorimetric procedure, but for this study the fluorometric hexokinase method above was used. Small aliquots of hemolymph, both directly and deproteinized and hemolymph concentrated by lyophilization were tested (Appendix E).
Hemolymph Extraction and Fractionation Procedures

The various fractionation procedures used are presented in flow diagram form in Figure 2. The 70% ETOH extraction procedure was done as a preliminary study. Hemolymph was sampled as above and the remaining tissue homogenized with 70% ETOH or 10% TCA (trichloracetic acid). The anthrone determination was then done on the free carbohydrate fraction, the polysaccharide fraction and the acid-base treated free carbohydrates fraction from both extraction procedures used on both hemolymph and tissues. On the basis of the results discussed below, the 10% TCA-ETOH procedure was used throughout the study. Only TCA-ETOH extracts, free of polysaccharides, were used for the acid-base hydrolysis (A-B CHO) since, although polysaccharides are hydrolyzed, it is generally at a slow rate, and would give a large positive anthrone reaction, if not removed.

The deproteinization procedure used is the Somogyi method, to remove protein, particularly extraneous enzymatic activity in the enzymatic assays of glucose and trehalose (Somogyi, 1930; Figure 2 D). The neutrality of the combined Ba(OH)$_2$-ZnSO$_4$ reagent was checked by titrating 5 mls of ZnSO$_4$ with Ba(OH)$_2$ using phenolphthalein as an indicator (Sigma Bulletin No. 510).
Figure 2

FRACTIONATION EXTRACTION PROCEDURES

A. 70% ETOH EXTRACTION

Hemolymph or Tissue

70% w/v ETOH

Extract / Homogenize

15 min. R.T.

Centrifuge

15 min. 2600 RPM

Precipitate

Tissue
Discard

Hemolymph

Dissolve in dist. H2O

Anthrone

PCHO

Supernatant

Evaporate to dryness

Take up in known volume of dist. H2O

Centrifuge

2600 RPM 10 min.

Precipitate

Dissolve in dist. H2O

Anthrone

PCHO

Supernatant

Anthrone

FCHO
B. TCA-ETOH EXTRACTION

Hemolymph or tissue

1:1 v:v, w:v 10% TCA extract

10 minutes R.T. Centrifuge

Precipitate
Discard

Supernatant
2 volumes 95% ETOH
0.1 volume 1 M NaCl

Refrigerate
5°C
3 h to overnight

Centrifuge

Precipitate
Dissolve in dist. H₂O
Anthrone

Supernatant
Anthrone
FCHO

PCHO
C. ACID-BASE HYDROLYSIS

FCHO Fraction

Evaporate to dryness

Air stream + warm sand bath (65°C)

Acid

Add 0.2 ml 0.1 N HCl per 20-100 μg CHO
loose cap test tube
10 min boil water bath
Remove from bath

Base

Add 0.15 ml 6 N NaOH per 20-100 μg CHO
Recap
10 min boiling water bath

Remove tubes

Cool to R.T.

Adjust volume to 1 ml

Dist. H₂O

Anthrone

A-B CHO
**Figure 2**

**D. Ba(OH)$_2$-ZnSO$_4$ Deproteinization**

Hemolymph (1 volume)  
Mix well  
2 x hemolymph volume  
Ba(OH)$_2$  
Mix well  
2 x hemolymph volume ZnSO$_4$  
(Adjusted so Ba(OH)$_2$ - ZnSO$_4$  
Centrifuge  
20 min  
2600 RPM  
Supernatant  

---

Enzymatic CHO Determination  
Electric Desalting Apparatus  
check % and ammeter  
Lyophilize  
Take up in pyridine  
TLC
Thin-Layer Chromatography

Thin-layer chromatography (TLC) was done as a second method to characterize oyster hemolymph. The method is primarily qualitative, but is capable of giving detailed information as to what specific carbohydrates are present.

The general methodology followed is that presented by Stahl (1969). More specifically, the methods used for crustaceans and insects were used as a starting point for development of the techniques (Boctor and Sale, 1973; Moriarity, 1976; Telford, 1965, 1968 b,c; McWinnie and Sailer, 1960). For the purposes of discussion the TLC process will be divided into four stages: extraction of hemolymph, desalting, spotting and development, and visualization.

I. Extraction

The two major problems encountered in preparing hemolymph carbohydrates for TLC were: to obtain enough material, that on development the spots could be seen, and to obtain a preparation that was mobile on the TLC plates.

Primarily, two extraction-deproteinization procedures, combined with various desalting methods, were tried. The first was the standard TCA-ETOH method and the other was the Somogyi (1930) method. A few samples of hemolymph were placed in boiling water for 30 seconds and centrifuged. The final supernatants from these procedures were used directly, or concentrated by lyophilization, if necessary, and then used with the various desalting methods (Figure 2 B and D).
II. Desalting Techniques

The "batch" method of deionization was tried on TCA-ETOH FCHO extracts and acid-base treated FCHO using Bio Rad Laboratories (Richmond, California) resin AG-11 A8 (Bio Rad Tech. Bull. 1005). This resin has both anion and cation exchange groups bound to the same resin structure, the groups being self absorbed. The technique consists of placing a 1:1 volume of sample and resin in a capped centrifuge tube with constant gentle agitation. The salt content for this and other procedures was followed by using an A/O Refractometer calibrated for salinity. Only a 50 µl sample is necessary.

Bio Rad Laboratories also suggests the resin can be used as an ion-cation mixed bed ion exchange resin or as an ion-retardation resin for separation of ions and neutral molecules. This suggestion was tested using a 1 ml sample of 1 mg glucose in 16 °/oo artificial sea water with glass distilled water as the eluant. Glass columns 0.9 x 25, 1.1 x 25 and 1.1 x 49 cm, with flow rates of 6 ml/hr and 12 ml/hr, were used. One ml fractions were collected of which 0.5 ml was used for an anthrone determination and salinity was determined with the refractometer.

Samples of the TCA-ETOH FCHO and the Somogyi supernatant were run on Dowex ion exchange resins. Small columns of Dowex 50 W x 2 (H⁺ form) and Dowex 1 x 4 100 (converted to the CO₃⁻ form by passing 40 volumes of 2 M Na₂CO₃ through it). The columns were prepared in 5 ml disposable pipets, containing 2-4 mls of resin bed. The sample was passed through the cation exchanger, then the anion
exchanger with distilled water as the eluant. Ten mls of eluate were collected and lyophilized (Telford, 1965; Bio Rad Laboratories Technical Service, personal communication).

An electrochemical desalting device, (Research Specialties, Inc., Richmond, California) designed specifically for removing salts for paper chromatography preparations was tried. The device consists of a chamber containing mercury, which acts as the cathode on which the sample is floated. A second glass chamber is lowered until the dialysis membrane covering its orifice just touches the sample. This chamber contains the anode and an electrolyte solution (6 mls H₂SO₄/liter distilled water) which is renewed from a reservoir at a slow rate. A water lift keeps the mercury in the lower chamber constantly stirred. A potential is placed across the electrodes. The anions move across the dialysis membrane where they are flushed away, while the cations are amalgamated with the mercury. An ammeter allows monitoring of the process because, as the salts are removed the current across the electrodes drops. The sample was also checked with the refractometer for "salinity". Samples were not removed until either the refractometer reading was 0 °/oo or, if close to zero, a further 30 minutes in the apparatus neither lowered the ammeter or the refractometer reading. The samples are then removed from the chamber and lyophilized.

III. Spotting and Development

A known amount of the lyophilizate from the above extraction desalting process was taken up in water: ETOH
(2:1) or pyridine (Telford 1968 b). Generally a volume of 5-20 μl of material was spotted onto the plate. Standard sugars were also spotted, approximately 8-10 μg per carbohydrate. Sample spotting is done with 5 or 10 μl micro-pipets. The plate is warmed with a heat lamp, held approximately one foot away to aid solvent removal. The plate was then allowed to air dry at least an additional 15 minutes. All chromatograms were developed in "sandwich" type chambers (Stahl, 1969) either from a commercial source (Eastman Kodak Co.) or made from window plates with cardboard spacers and clamps (A. Thomas Scientific, Philadelphia, Pa.).

Only E. M. Merck prepared plates on aluminum backing were used. Silica gel plates, type F-64 0.2mm thick, without fluorescent indicator, were generally used, but cellulose plates were tried as well.


Generally, solvents, TLC plates, and the various visualization methods were tested using 10 μg samples each of glucose, trehalose, glucose-1-phosphate, maltose and galactose standards. When experimental materials were used, generally two samples or sample duplicates were spotted on each TLC plate.
IV. Visualization

After development plates were air dried in a hood overnight. If appropriate the plates were divided into various strips for different treatments. Color reagents were applied using individual jars with an interchangeable spray propellant head and manifold.

Spraying was done in a standard hood with a cardboard box to act as a spray cabinet. Care was taken to cover the plate uniformly. If necessary, plates were heated in a laboratory oven at 100-110°C for the appropriate time period, but often checked every 5-10 minutes. Occasionally, if color development seemed poor, the plate was sprayed and heated. The spray reagent list appears in Appendix F.
COLORIMETRIC RESULTS

Evaluation of Extraction Techniques for Hemolymph Carbohydrates

The 70% ETOH and TCA-ETOH extraction methods, (Figure 2 A, B), were evaluated on the hemolymph and tissues of four individual oysters (Figure 1, Table 2). The two methods are about equally effective in extracting the tissue FCHO, however, the TCA-ETOH method has the advantage of giving a "clean" polysaccharide fraction (PCHO) as well.

For the hemolymph, the precipitate from the 70% ETOH extract was reserved since it appeared to be a clean white material similar to that of the TCA-ETOH precipitate. In fact, throughout the study the initial TCA protein precipitate never yielded more than a very slight skim of material in the centrifuge tube. This would imply that there is very little protein in the hemolymph.

Both the FCHO and PCHO values for the hemolymph TCA-ETOH extraction are higher than the hemolymph 70% ETOH extraction with the free carbohydrate being much greater than the 70% ETOH FCHO. The reason for the difference in the FCHO fractions is not a simple partitioning of material, as the amount of carbohydrate not in the 70% ETOH FCHO fraction does not appear in the PCHO fraction. On the basis of giving higher values, as well as giving a
### TABLE 2
Comparison of Carbohydrate Extraction Methods

<table>
<thead>
<tr>
<th>Fract.</th>
<th>Tissues</th>
<th>Hemolymph</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FCHO&lt;sup&gt;1&lt;/sup&gt;</td>
<td>PCHO&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>70% ETOH</td>
<td>TCA-ETOH</td>
</tr>
<tr>
<td>Oyster</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1330</td>
<td>630</td>
</tr>
<tr>
<td>2</td>
<td>1162</td>
<td>1650</td>
</tr>
<tr>
<td>3</td>
<td>1212</td>
<td>1475</td>
</tr>
<tr>
<td>4</td>
<td>1937</td>
<td>1725</td>
</tr>
<tr>
<td>Avg.</td>
<td>1410</td>
<td>1370</td>
</tr>
</tbody>
</table>

1 µg.CHO/g. wet wt.
2 mg.CHO/g. wet wt.
3 µg.CHO/ml.
"clean" polysaccharide fraction, the TCA-ETOH extraction was used for this study.

It was noted that the polysaccharide obtained from hemolymph was quite different in appearance from that obtained from the tissues. The latter flocculates immediately and completely on addition of 95% ETOH to the solution. On centrifugation it forms a hard, opaque, white pellet. Most of this material is assumed to be glycogen. The hemolymph material flocculates more slowly, therefore an overnight, refrigerated precipitation was used. On centrifugation a rather soft gelatinous pellet with a clear white appearance was formed, which does not completely redissolve in the same volume of distilled water as the starting hemolymph volume. Due to its appearance and solubility, the hemolymph carbohydrate is probably different from the tissue polysaccharide in monosaccharide composition, structure, and molecular weight.

It is interesting that the free carbohydrate for tissue is much higher than that for hemolymph. However, the total of the free plus polysaccharides values for hemolymph is reasonably close to the free carbohydrate value for tissue. This would be consistent with the concept that, except for the glycogen content, the intracellular composition is similar to the extracellular and that the extracellular fluid is close to being 50% of the tissue by wet weight as estimated by other methods (Martin et al, 1958).
Comparison of Total APS, FCHO, and PCHO in Hemolymph From Field Samples

The data from the field survey for each location and analysis of the pooled hemolymph and individual animals appear in Table 3 and Figures 3 and 4.

The values for the total APS on either a wet weight and dry weight basis, respectively, varied greatly at each location: 150 to 2750 µg/ml, 5.42 to 16.5 g wet wt, and 1.2 to 3.5 g dry wt for the Eastern Shore, 770 to 2920 µg/ml, 14.9 to 29.1 g wet wt and 1.95 to 5.98 g dry wt for VIMS and 60 to 3400 µg/ml, 16.2 to 28.4 g wet wt and 2.2 to 5.5 g dry wt (Figures 3 and 4) for the Rappahannock River. There is as much or more variation between individuals of approximately the same weight as between individuals of different weight (Figures 3 and 4). Although there is some clustering of values from the same sample location, there is considerable overlap. Not unexpectedly the Eastern Shore values form a slightly separate group, but whether this is due to size difference, salinity or some other reason is unknown.

Some of the variability in the individual total APS values may be due to differing responses to handling by individual oysters. Hyperglycemic responses to handling, during sampling, has been reported for other invertebrates (Telford, 1968a; Marques and Falkner, 1976). Contamination of an occasional sample with shell fluid could cause an outlying value, but this seems unlikely. The data from the extraction method study indicates the uniformity of the
<table>
<thead>
<tr>
<th>Sample</th>
<th>Total CHO</th>
<th>FCHO</th>
<th>PCHO</th>
<th>A-B CHO</th>
<th>Avg. Wet Weight</th>
<th>Avg. Dry Weight</th>
<th>Total CHO/ g. wet wt.</th>
<th>Total CHO/ g. dry wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 Pooled</td>
<td>1420</td>
<td>189</td>
<td>682</td>
<td>65</td>
<td>19.6</td>
<td>4.2</td>
<td>72</td>
<td>338</td>
</tr>
<tr>
<td>25 Individuals</td>
<td>1847</td>
<td></td>
<td></td>
<td></td>
<td>21.7</td>
<td>3.9</td>
<td>95</td>
<td>511</td>
</tr>
<tr>
<td>Sx</td>
<td>132</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rappahannock R.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 Pooled</td>
<td>1245</td>
<td>222</td>
<td>380</td>
<td>28</td>
<td>19.0</td>
<td>3.7</td>
<td>66</td>
<td>336</td>
</tr>
<tr>
<td>25 Individuals</td>
<td>933</td>
<td></td>
<td></td>
<td></td>
<td>21.3</td>
<td>4.1</td>
<td>46</td>
<td>230</td>
</tr>
<tr>
<td>Sx</td>
<td>127</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eastern Shore</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 Pooled</td>
<td>1080</td>
<td>238</td>
<td>418</td>
<td>56</td>
<td>10.0</td>
<td>2.2</td>
<td>108</td>
<td>491</td>
</tr>
<tr>
<td>25 Individuals</td>
<td>1209</td>
<td></td>
<td></td>
<td></td>
<td>10.2</td>
<td>1.9</td>
<td>123</td>
<td>637</td>
</tr>
<tr>
<td>Sx</td>
<td>136</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1All values as µg anthrone positive substances/ml of hemolymph fractions as in methods
sampling procedure (Table 2). The similarity between values for pooled hemolymph and the average of the individually analyzed samples, discussed below, further indicates that hemolymph sampling from successive groups of twenty five oysters was done in a uniform manner.

The analysis of the pooled hemolymph for total APS, FCHO, PCHO, and A-B CHO shows that the hemolymph composition is quite similar regardless of the geographic origin of the oysters (Table 3). The composition is similar not only in the relative proportions of the carbohydrate fractions, but in the absolute concentration (µg/ml) values as well. The total APS values were normalized for dry weight, for comparison, as the Eastern Shore animals were about half the size as those from the other two locations. The values are quite similar, at all geographical locations, whether total APS is compared for the average of the individual hemolymph samples, the pooled hemolymph total APS analysis, or normalized for weight. This suggests that either method of analyzing hemolymph is valid for determination of total APS, regardless of animal size or geographical location.

Seasonal Variation in Total APS, FCHO, PCHO and A-B CHO in Field and Laboratory Oysters

Although a seasonal experiment was not planned, various samples of hemolymph were taken from oysters held at VIMS for other assay procedures (Figure 5). As a baseline, samples were always analyzed for total APS, FCHO, and PCHO. It should be cautioned, however, in drawing con-
elusions from these data that neither the time interval between samples nor the number of animals used for each sample was uniform. It also should be noted that the concentration change could be due to either change in the absolute amount of carbohydrate or changes in the size of the total fluid compartment (Dall, 1974). The summary (Table 4) presents the concentration data for the various fractions which has been plotted versus time (Figure 5). The most striking result is the drastic drop and then rise in the total APS which is primarily due to changes in PCHO. This is partly because the PCHO is a large proportion of the total, but also because the change is quite large, the concentration changing from 800 μg/ml to 63 μg/ml a whole order of magnitude. The FCHO and A-B CHO also varied greatly, by almost an order of magnitude, but these fractions are a relatively small proportion of the total and did not have as great an effect on the total APS value.

The concentration values were recalculated as a percentage of the total CHO and plotted versus time (Figure 5, B). All the fractions remain relatively stable during the winter, then at different times in the spring they show a rapid decline. Again the PCHO had the most dramatic change, while the FCHO was most stable. Interestingly the declines in concentration follow one another with the PCHO occurring first, the A-B CHO next and finally the FCHO. This would imply that the first fractions are catabolized to maintain the FCHO.
Figure 5. Time variation in hemolymph carbohydrate and environmental factors:

A. μg of total anthrone positive substances
   * = total APS, 0 = PCHO, + = FCHO,
   X = A-B FCHO

B. Carbohydrate fractions as % of total APS
   0 = PCHO, + = FCHO, X = A-B FCHO

C. Water temperature (VIMS 1977)
   Arrows indicate 9°C, beginning of feeding, 16°C, beginning of gametogenesis

D. Tissue glycogen content
   X Glycogen as % wet weight (Walne, 1970)
   0 Glycogen in mg/g wet wt (Badman, 1967)
   + Condition index, arbitrary units using % scale (Haven, 1960)

E. Phytoplankton (After Manzi, 1973)
   * Diatoms
   0 Dinoflagellates
   + Unidentified phytoplankters
CHO FRACTIONS AS % TOTAL ANTHYrone POSITIVE SUBSTANCES/CELLS /ML.

- 1400 -
- 1200 -
- 1000 -
- 800 -
- 600 -
- 400 -
- 200 -
- 0 -
- 20 -
- 40 -
- 60 -
- 80 -
- 100 -
- 120 -
- 140 -

- JAN
- FEB
- MAR
- APR
- MAY

mg/g wet weight
### TABLE 4

Summary of Colorimetric Data for Hemolymph Carbohydrates

µg of CHO / ml oyster hemolymph

<table>
<thead>
<tr>
<th>Sample (Table 1)</th>
<th>Total CHO</th>
<th>FCHO</th>
<th>PCHO</th>
<th>A-B CHO</th>
<th>Hexosamine</th>
<th>Glucose (Glucose Oxidase)</th>
<th>Glucose Trehalose (HK-NADPH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan 23 Avg. 4 Ind.</td>
<td>1118¹</td>
<td>271</td>
<td>847</td>
<td>212</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>March 1 3 Pooled</td>
<td>1120</td>
<td>391</td>
<td>813</td>
<td>242</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>March 17 10 Pooled</td>
<td>605</td>
<td>191</td>
<td>190</td>
<td>118</td>
<td>18</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>April 1 Avg. 4 Ind.</td>
<td>234</td>
<td>87</td>
<td>63</td>
<td></td>
<td></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>May 2 10 Pooled</td>
<td>890</td>
<td>240</td>
<td>250</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>May 16 E. Shore 25 Pooled</td>
<td>1080</td>
<td>238</td>
<td>418</td>
<td>56</td>
<td></td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>May 18 VIMS 25 Pooled</td>
<td>1420</td>
<td>189</td>
<td>682</td>
<td>65</td>
<td></td>
<td></td>
<td>22 0.12</td>
</tr>
<tr>
<td>May 31 Rappahannock 25 Pooled</td>
<td>1245</td>
<td>222</td>
<td>380</td>
<td>28</td>
<td></td>
<td></td>
<td>54</td>
</tr>
</tbody>
</table>

¹Sum of FCHO and PCHO; all other values determined independently
Specific Enzymatic Colorimetric Methods for Glucose and Trehalose

I. Glucose Oxidase - Glucose Determination

The glucose oxidase method did measure glucose in oyster hemolymph. However, the test is unsuitable as the levels of glucose found were well below the detection limit for the method, as indicated by the following set of experiments.

In the first experiment, done March 23, hemolymph sampled on March 17 (Table 1) was used. Each sample was done in duplicate; the data appears in Table 5, Experiment I. Samples included were hemolymph without any treatment, hemolymph spiked with 100 mg% of glucose, deproteinized hemolymph and hemolymph spiked with glucose and then deproteinized (concentration adjusted so final was 100 mg%). Both the untreated and deproteinized samples gave a small positive result of 1 and 2.2 mg% (10 and 22 µg/ml) respectively. Only the spiked sample gave a number very different from 0 mg% being 12 mg%, after correction for the spike. The average of the four determinations was 4 mg%.

The second experiment was done to test if, during the incubation, breakdown of glycogen occurred thereby inflating the glucose values (Table 5, Experiment II). The incubations were done on March 30 using hemolymph sampled on March 17 (Table 1). The incubations of hemolymph sample were carried out at room temperature for 60 minutes in duplicate, then the enzyme reagent added and the assay done
in the usual manner. The first sample consisted of hemolymph incubated for 60 minutes, followed by addition of the glucose spike and the enzyme. The second sample, consisted of hemolymph and the glucose spike incubated 60 minutes, followed by addition of the enzyme, to check loss of glucose during the incubation. The third sample contained hemolymph and 100 mg% glycogen incubated for 60 minutes and followed by addition of the glucose spike and enzyme. The final sample was hemolymph, glucose spike, and enzyme done as usual. A glucose standard and a standard containing glycogen were run. The standard with glycogen gave the same absorbance value as the glucose standard, indicating that there is no breakdown of glycogen by the enzyme reagent. All the experimental values were within the range expected for pipetting errors of 100 mg% sample replication. Therefore, it was concluded that there was no significant loss of glucose on prolonged incubation or production of glycogen. The average for the determinations was 100.25%, giving a hemolymph value of essentially zero.

A third experiment was done on April 1 using freshly sampled hemolymph from four individual oysters (Table 1). The hemolymph was frozen and thawed and treated in much the same way as in the first experiment; a determination was done on the deproteinized hemolymph with glucose spike. The experiment was done to check if previous low values might be due to loss of glucose during storage. The freshly drawn hemolymph gave essentially identical results to the other two experiments (Table 5, Experiment III).
<table>
<thead>
<tr>
<th>Experiment-Conditions</th>
<th>Absorbance 1</th>
<th>Absorbance 2</th>
<th>Avg. mg% Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Hemolymph</td>
<td>0.00</td>
<td>0.005</td>
<td>1</td>
</tr>
<tr>
<td>2. Hemolymph+100mg% glucose</td>
<td>0.245</td>
<td>0.250</td>
<td>112</td>
</tr>
<tr>
<td>3. Deprotn. Hemolymph</td>
<td>0.008</td>
<td>0.005</td>
<td>2</td>
</tr>
<tr>
<td>4. Deprotn. Hemolymph+100mg% glucose</td>
<td>0.290</td>
<td>0.292</td>
<td>100</td>
</tr>
<tr>
<td>5. Standard</td>
<td>0.290</td>
<td>0.292</td>
<td>100</td>
</tr>
<tr>
<td><strong>Experiment II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Hemol. + Glucose + Enzyme</td>
<td>0.265</td>
<td>0.245</td>
<td>98</td>
</tr>
<tr>
<td>45 min. incubation then read</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Hemolymph</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 min. incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose + enzyme, 45 min. incub. then read</td>
<td>0.255</td>
<td>0.255</td>
<td>98</td>
</tr>
<tr>
<td>3. Hemol. + Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 min. incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme, 45 min. incub., read</td>
<td>0.272</td>
<td>0.260</td>
<td>102</td>
</tr>
<tr>
<td>4. Hemol. + Glycogen (100 mg%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 min. incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose + enzyme, 45 min. incub., then read</td>
<td>0.268</td>
<td>0.280</td>
<td>103</td>
</tr>
<tr>
<td>5. Standard + Glycogen</td>
<td>0.260</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Standard (100 mg% glucose)</td>
<td>0.260</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment-Conditions</td>
<td>Absorbance</td>
<td>Avg. mg% Glucose</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>-------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Experiment III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh Hemolymph</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Individual Oysters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemolymph:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.006</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0.018</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Hemolymph + 100 mg% glucose:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.175</td>
<td></td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>0.172</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>0.178</td>
<td></td>
<td>99</td>
</tr>
<tr>
<td>4</td>
<td>0.190</td>
<td></td>
<td>107</td>
</tr>
<tr>
<td>Deproteinized hemol. + 100 mg% glucose:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.112</td>
<td>0.116</td>
<td>102</td>
</tr>
<tr>
<td>2</td>
<td>0.110</td>
<td>0.108</td>
<td>97</td>
</tr>
<tr>
<td>3</td>
<td>0.108</td>
<td>0.114</td>
<td>99</td>
</tr>
<tr>
<td>4</td>
<td>0.108</td>
<td>0.102</td>
<td>94</td>
</tr>
</tbody>
</table>
Only four values are different from zero; 3 and 10 mg% for raw hemolymph, 107 mg% for hemolymph with glucose, and 102 mg% for the deproteinized hemolymph. The average result was 3 mg% for the untreated hemolymph, 100 mg% for the spike and 98 mg% for the deproteinized and spiked material. The last two values are equivalent to zero for the hemolymph glucose.

The glucose oxidase method has limitations for measuring glucose in oyster hemolymph. This is not because of loss of glucose, interfering substances, or release of polysaccharide glucose. The consistent and essentially 100 percent recovery of the glucose spike indicated that the method will measure glucose if present. The glycogen incubations demonstrate that there is not interference from this material either in increasing or decreasing the true value. The problem is that oyster hemolymph glucose concentration is at or below the detection limit for the method. The average value found was 0 to 4 mg%, which is well below the estimated limit of 12 mg% from the standard curve or the suggested limit of 20 mg% for clinical work (Sigma Technical Bulletin No. 510).

II. Hexokinase-NADPH Glucose Determination

The HK-NADPH method using the fluorometric technique was used to obtain a more accurate value for glucose, as it is considerably more sensitive than the glucose oxidase method. The range in values for the determination was 0.3-10 mg% with an average of 4 mg%, which is consistent with the value for the glucose oxidase method.
The basic procedure followed is described in Appendix D. Hemolymph used was from the pooled samples from VIMS, the Eastern Shore, and the Rappahannock River (Table 1). For each experiment glucose standards of 10 mg%, hemolymph, and hemolymph with a 10 mg% glucose spike were done in triplicate with three fluorometer readings taken for each replicate (Table 6).

The reproducibility of the fluorometer readings was generally good. The percentage recovery values indicate that there is little loss of glucose. The high values for the Eastern Shore might indicate some release of glucose by the enzyme assay reagents, but this is unlikely. The hemolymph glucose values with the spike, once corrected, usually gave slightly higher numbers than the hemolymph directly. This is probably due to a decrease in errors with the higher fluorometer readings obtained with the spiked sample. Again the problem seems to be that the hemolymph glucose concentrations are close to the limit of detection and reproducibility for the method which is 1 mg%.

III. Enzymatic Determination of Trehalose

The enzymatic assay for trehalose gave extremely low values and variable results, with an average of 0.12 μg/ml (Table 4). This value is a third of the glucose value, which is quite low compared to other determinations. The reason for this low glucose value is unclear. Interestingly this trehalose value is much less than either the A-B CHO, which had tentatively been designated as trehalose, or the values reported for whole tissue extracts, 0.5 to
### TABLE 6
Hexokinase-NADPH Determination of Hemolymph Glucose

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hemolymph mg% glucose</th>
<th>Hemolymph + glucose 10 mg%</th>
<th>% Glucose recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIMS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.8</td>
<td>14.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.7</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.2</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>Avg.</td>
<td>3.9</td>
<td>12.7</td>
<td>88</td>
</tr>
<tr>
<td>E. Shore</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.2</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.0</td>
<td>16.1</td>
<td></td>
</tr>
<tr>
<td>Avg.</td>
<td>0.73</td>
<td>14.2</td>
<td>135</td>
</tr>
<tr>
<td><strong>Experiment II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIMS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.38</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.15</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.38</td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td>Avg.</td>
<td>0.30</td>
<td>12.0</td>
<td>117</td>
</tr>
<tr>
<td>E. Shore</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8.0</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8.0</td>
<td>19.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6.0</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>Avg.</td>
<td>7.0</td>
<td>20.0</td>
<td>130</td>
</tr>
</tbody>
</table>
TABLE 6 (Continued)
Hexokinase NADPH Determination of Hemolymph Glucose

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hemolymph mg% glucose</th>
<th>Hemolymph + glucose 10 mg%</th>
<th>% Glucose recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rappahannock R.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.7</td>
<td>14.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.7</td>
<td>14.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.7</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>Avg.</td>
<td>4.7</td>
<td>15.0</td>
<td>103</td>
</tr>
</tbody>
</table>
2 μg/g wet weight (Badman, 1967).

The basic procedure used is in Appendix E. The pooled sample taken at VIMS on May 18 was used for all assays (Table 1). Two initial experiments were done with 10 μl of hemolymph but were unsuccessful. In both cases the fluorometer values gave impossibly high values for glucose and trehalose close to the total APS, and in one case the percentage recovery was less than zero. A second experiment was tried using 200 μl hemolymph, which was deproteinized and then lyophilized, to eliminate any enzymes which might interfere with the assay. No glucose value could be obtained for any of the samples, including the standards. It was concluded that the residual Ba-Zn from the deproteinization, by being concentrated, poisoned the glucose assay system.

Three experiments using 200 μl of lyophilized hemolymph were done to concentrate any trehalose present and obtain a more accurate value from the assay. A blank, glucose standard, trehalose standard, hemolymph without trehalase (for native glucose), hemolymph with trehalase (native glucose plus glucose from trehalase hydrolysis) and hemolymph with trehalase and a trehalose spike (to determine percent recovery) were run for each experiment. The mg% values, micromoles of glucose and trehalose as well as trehalase activity in units, and percentage recovery are reported (Table 7).

In experiment 3 the percentage recovery of
# TABLE 7

**Enzymatic Assay of Trehalose**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>µg/ml Trehalose</th>
<th>Glucose (µm)</th>
<th>Units (µmoles trehalose hydrolyzed/hr)</th>
<th>% Recovery of Trehalose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.02</td>
<td>0.29</td>
<td>$0.44 \times 10^{-2}$</td>
<td>47</td>
</tr>
<tr>
<td>2</td>
<td>0.33</td>
<td>0.43</td>
<td>$2.4 \times 10^{-2}$, $0.4 \times 10^{-2}$ (maltose)</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>0.0075</td>
<td>0.36</td>
<td>$1.5 \times 10^{-2}$, $0.35 \times 10^{-2}$ (maltose)</td>
<td>0</td>
</tr>
</tbody>
</table>
trehalose was zero and the value from the previous determination used. This points out a major problem encountered with the assay. The amount of glucose produced from either native trehalose or the spike was quite small relative to the glucose already present. The glucose value itself was quite low, near the detection limits of the HK-NADPH system. Therefore, very small errors or variations in the individual samples could produce widely varying and inconsistent results.

A second problem is the low recovery rate of the trehalose spike and the low value, compared to previous determinations, for hemolymph glucose. Since a glucose spike was not introduced, it can not be determined whether the loss is due to loss of trehalose or failure to detect glucose as it is produced in the incubation. However, due to the low glucose values and percentage recovery it seems clear that there is considerable interference from the hemolymph. If this problem could be defined and eliminated the assay would improve; by increasing the absolute colorimetric value measured, making the results less variable.

The trehalase preparation was assayed twice for maltase activity by doing incubations with 3 micromoles of maltose. The units obtained were $0.4 \times 10^{-2}$ and $0.35 \times 10^{-2}$, which is ten times that reported (Hey and Elbein, 1968). This is unfortunately a high (20%) proportion of the trehalase activity. Therefore, the values for trehalose are suspect, for at least some of the reported value is due to maltose and the most extreme interpretation, all of it
being maltose, is possible. Since it was not possible to obtain an independent value for maltose concentration, it is impossible to determine what portion of the value is trehalose or maltose.

Summary of Carbohydrate Composition of Oysters Based on Colorimetric Tests

The values from the various assay procedures are summarized here to obtain an overview of the carbohydrate composition of oyster hemolymph (Table 4).

The fact that polysaccharides (PCHO) were such a large proportion of the total APS and varied widely was an unexpected result. The free carbohydrate (FCHO) proportion was relatively constant. It was initially assumed that most of this FCHO would be glucose. The level of glucose however, accounts for a very small portion of the free carbohydrate total, even when the A-B CHO is not considered. For example, the sample taken at VIMS on May 18 had a total FCHO value of 189 μg/ml. This becomes 124 μg/ml when the A-B CHO is subtracted, but the authentic glucose assay gives a value of 40 μg/ml, leaving a large amount of the FCHO unidentified (Table 4).

The A-B CHO itself is a large, but variable proportion of the FCHO. Originally this assay was done as a test for trehalose, since it was expected that polysaccharides, trehalose, and chitin or its precursors would be the only carbohydrates not hydrolyzed by this process. The polysaccharides were known to be eliminated by the TCA-
ETOH fractionation, which is considered to be very "clean". Since glucosamine is a major constituent of chitin, the hexosamine assay was done to estimate this material (Appendix B). Even if all the hexosamine found, 18 to 22 μg/ml, were from chitin, it can only account for 10 to 15% of the anthrone positive substances in the A-B CHO. Therefore it was expected that most of this material was trehalose. The TLC and enzymatic assay did not substantiate this conclusion. The specific trehalose assay value of 0.12 μg/μl, while perhaps not accurate, is probably of the right order of magnitude and is two orders smaller than the A-B CHO.
THIN-LAYER CHROMATOGRAPHY RESULTS

The various chromatograms showing the TLC results are presented in Figures 6 and 11. For clarity, plates are presented as tracings of the originals with solvent systems, development time, identification of spots, mobilities in centimeters and hRf (dist spot/dist solvent front x 100) listed on the opposing page. Designation of unknown spots on the figures is by sample number at the origin of the TLC plate and alphabetic lettering from the upper most spot on the plate down. Unless otherwise stated, the visualization reagent was naphthoresorcinol. Due to slight differences in conditions, such as concentration, residual salts, presence and amount of interfering substances, mobilities of an individual material can vary ± 0.25 cm. This level of resolution causes problems in absolute identification of unknowns, since carbohydrates with similar molecular weight and functional groups often have very similar mobilities.

Evaluation of Several Extraction and Desalting Techniques

Considerable difficulties were encountered in obtaining extracts with sufficient material for visualization and with sufficient mobility for proper development. In preliminary studies, using extracts from the 70%
ETOH and TCA-ETOH comparison study and the acid-base treated material, either no spot was visualized or a positive reaction was obtained only at or near the origin. A set of glucose and trehalose standards was run with varying amounts of artificial sea water (Figure 6) to test the salting effect. The salt influenced the mobility of the trehalose most, but even a small amount, 1.8 °/oo, alters mobilities from that of the standard. Therefore, a variety of extraction and desalting methods were tried.

Preliminary trials of desalting extracts with the Bio Rad resin AG 11 were unsuccessful. In the batch method, even after 18 to 24 hours, there was no decrease in salt content as measured by the refractometer. The column method also failed for although some separation of the carbohydrate and salt peaks occurred, there was an estimated 30 to 50% overlap. Varying column size, flow rates and consultation with Bio Rad's Technical Service failed to solve the problem.

Samples from all the extraction procedures were tried using both ion exchange columns and the electric desalter. The first problem encountered with the ion exchange columns was bubble formation in the anion exchange bed. This seemed to be due to the formation of CO₂ as the H⁺ was released from the cation exchanger, and combining with the anion CO₃⁻. Not all samples produced this effect or produced it consistently. This problem might be solved by carefully buffering the sample and eluting with a buffer. Eluents that were collected, concentrated and spotted generally showed little or no carbohydrate present (Figure 7, Nos. 2 and 3).
Trials were performed with the same extracts but using the electric desalting apparatus (EDS). The preliminary results seemed promising so the column approach was abandoned. A typical example of comparable results is shown in Figure 7. A sample of hemolymph was treated four different ways: TCA-ETOH extraction with EDS, boiled 30 seconds and run on ion exchange column, boiled 30 seconds and run on EDS, and Ba-Zn deproteinization followed by EDS. Only the electric desalted samples showed any material present, with more material being visualized from the Ba(OH)$_2$-ZnSO$_4$ deproteinization.

From this and similar experiments the combination of Ba(OH)$_2$-ZnSO$_4$ deproteinization followed by EDS and lyophilization was used for all subsequent work. It was found best to take up the lyophilizate in pyridine rather than water:ETOH. The latter samples showed little or no mobility even when there was a considerable positive carbohydrate reaction confined to the origin on the TLC plate. This is presumably due to elimination of any small amount of residual salt content from the samples as salts are not soluble in pyridine.

Problems of obtaining sufficient carbohydrate for good visualizations were not completely solved in this study. The desalted extracts on lyophilization yielded a fluffy residue, generally white but occasionally greyish, probably due to mercury adsorbed on the carbohydrate during the desalting process. Generally it was found necessary to take up 1 to 2 mg in 50 µl of pyridine with 10 to 20 µl being
spotted for adequate visualization. Despite the apparent large quantity of material dissolved and spotted it was estimated carbohydrate spots contained 0.5 to 5 μg of material. This would imply that a large portion of the carbohydrate in the extract was not mobile on the chromatograms. The most likely explanation is that it is polysaccharide.

Identification of Oyster Hemolymph Free Carbohydrates

One of the first successful chromatograms had hemolymph from several different sampling periods (Figure 8). The composition of the March 1 and 17 samples showed a slightly different pattern, but with two spots matching the others. Whether this difference is due to real seasonal changes in hemolymph composition or preparation technique is unknown. On the basis of inspection and hRF values identifications were made. The spot with hRF of 30 and 32 as glucose, hRF 21 as trehalose or maltose, leaving hRF 24 and 12 unidentified. The glucose-1-phosphate standard (Ba salt) did not go into solution in pyridine. Assuming the sample sugar phosphates form salts, which is likely, they would not chromatograph. Therefore, although the March 1 and 17 spot with hRF 12 might be glucose-1-phosphate it may be a neutral sugar with the same or nearly same mobility. Plates 118 and 119 (Figures 9 and 10) resolve the samples into four spots. The first spot co-chromatographed with glucose and fructose. Due to color quality and the aspect that glucose is much more common, it was designated as glucose.
The second spot, B, chromatographed with galactose, but not glucuronic acid on plate 118, whereas on plate 119 it co-chromatographed with one spot of glucuronic acid, so it was designated as galactose. The third spot, C, co-chromatographed with a part of glucuronic acid on plate 119 but with only maltose or trehalose on plate 118. On the additional basis of color it was designated as maltose, trehalose or a mixture of both. To resolve which one, half of plate 126 (Figure 11) was sprayed with naphthoresorcinol and one with benzidine reagent, which visualizes maltose but not trehalose. The third spot, C, on plate 126 co-chromatographed with maltose and trehalose, but the spot is much smaller on the benzidine treated portion. Both samples were from the same preparation and the same amounts were spotted on the plate. Since its size and intensity were diminished with benzidine from the naphthoresorcinol treatment, there are probably both carbohydrates present.

The fourth spot, D, co-chromatographed with glucuronic acid on plate 118, but not on plate 119. On plate 118 it also co-chromatographed with an unknown material in the maltose standard. It was found that the maltose contained enough maltotriose contamination to be visualized. Further verification for the standard unknown as being maltotriose is that the hRf for authentic material from 126 is similar. The best evidence is the similarity of the Rm and log mobility values (described below) for the unknown and authentic maltotriose standard. Therefore spot D was designated to be maltotriose.
A manipulation of the mobility data was done to aid identification. Plots of the Rm, where $Rm = \log (1/Rf)-1$ or log mobility in cm versus the number of monosaccharide units for a homologous series have been shown to form a straight line (Telford, 1968; McWinnie and Sailer, 1960; Hough, 1954). Sugars having different structures, molecular weight or functional groups will not fall on the line at the proper hexose size.

In this case glucose, maltose or trehalose and maltotriose form a series, each being one, two, or three glucose units respectively. Mobility, hRf, Rm and log mobility were calculated from plates 118, 119 and 123 (Table 8, Figure 12). The unknowns are plotted with a letter designation corresponding to that used in describing the plate. Different chromatograms and different solvent systems form parallel lines with one another, but are internally consistent. Note the "maltotriose" from plate 118 falls exactly at 3 units, with the same value as the authentic material. The values for the presumptive glucose maltose/trehalose and maltotriose fall at the appropriate number of hexose units, substantiating their identity as part of the glucose homologous series. The presumptive galactose, not surprisingly, does not fall exactly at one hexose unit, but closer to one than any other value.

Therefore it is concluded that glucose, galactose, maltotriose, maltose and probably trehalose occur in oyster hemolymph. Although no quantitative work could be done the
observed relative amounts are based on inspection of spot intensities with diphenylamine and naphthoresorcinol:maltotriose, glucose, maltose/trehalose, galactose.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mobility in cms.</th>
<th>hRf$^1$</th>
<th>Rm$^2$</th>
<th>log mobility$^3$</th>
<th>Unknown designation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plate 118</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Front</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>5.25</td>
<td>31</td>
<td>0.35</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>4.25</td>
<td>26</td>
<td>0.45</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>3.0</td>
<td>18</td>
<td>0.66</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Trehalose</td>
<td>3.0</td>
<td>18</td>
<td>0.66</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Maltotriose</td>
<td>1.5</td>
<td>9</td>
<td>1.0</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Gluc.-1-P</td>
<td>1.25</td>
<td>7</td>
<td>1.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>6.0</td>
<td>35</td>
<td>0.27</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td><strong>Unknown A</strong></td>
<td>5.75</td>
<td>34</td>
<td>0.29</td>
<td>0.76</td>
<td>Glucose</td>
</tr>
<tr>
<td>B</td>
<td>4.5</td>
<td>26</td>
<td>0.45</td>
<td>0.65</td>
<td>Galactose</td>
</tr>
<tr>
<td>C</td>
<td>3.0</td>
<td>18</td>
<td>0.66</td>
<td>0.48</td>
<td>Malt./Treh.</td>
</tr>
<tr>
<td>D</td>
<td>1.5</td>
<td>9</td>
<td>1.0</td>
<td>0.18</td>
<td>Maltotriose</td>
</tr>
<tr>
<td><strong>Plate 119</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Front</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>6.25</td>
<td>39</td>
<td>0.19</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>5.0</td>
<td>31</td>
<td>0.35</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Trehalose</td>
<td>3.75</td>
<td>23</td>
<td>0.52</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>3.5</td>
<td>22</td>
<td>0.55</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Gluc.-1-P</td>
<td>0.5</td>
<td>3</td>
<td>1.5</td>
<td>-0.30</td>
<td></td>
</tr>
<tr>
<td>Maltotriose</td>
<td>2.0</td>
<td>12</td>
<td>0.87</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td><strong>Unknown March 17</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A'</td>
<td>6.0</td>
<td>38</td>
<td>0.21</td>
<td>0.78</td>
<td>Glucose</td>
</tr>
<tr>
<td>B'</td>
<td>3.75</td>
<td>23</td>
<td>0.52</td>
<td>0.57</td>
<td>Malt./Treh.</td>
</tr>
<tr>
<td>C'</td>
<td>2.0</td>
<td>12</td>
<td>0.87</td>
<td>0.30</td>
<td>Maltotriose</td>
</tr>
<tr>
<td><strong>Unknown March 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>6.0</td>
<td>38</td>
<td>0.21</td>
<td>0.78</td>
<td>Glucose</td>
</tr>
<tr>
<td>B</td>
<td>5.0</td>
<td>31</td>
<td>0.35</td>
<td>0.70</td>
<td>Galactose</td>
</tr>
<tr>
<td>C</td>
<td>3.5</td>
<td>22</td>
<td>0.55</td>
<td>0.54</td>
<td>Malt./Treh.</td>
</tr>
<tr>
<td>D</td>
<td>2.0</td>
<td>12</td>
<td>0.86</td>
<td>0.30</td>
<td>Maltotriose</td>
</tr>
<tr>
<td><strong>Plate 123</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Front</td>
<td>15.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>5.25</td>
<td>34</td>
<td>0.29</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>4.5</td>
<td>29</td>
<td>0.39</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Trehalose</td>
<td>3.0</td>
<td>19</td>
<td>0.63</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>2.75</td>
<td>18</td>
<td>0.66</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Maltotriose</td>
<td>1.5</td>
<td>10</td>
<td>0.95</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Gluc.-1-P</td>
<td>1.25</td>
<td>8</td>
<td>1.1</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>
Table 8 (Continued)

Thin-Layer Chromatography Mobility Data

1 \( hRf = \text{cm spot/cm front} \)

2 \( Rm = \log \left( \frac{1}{Rf} \right) - 1 \)

3 \( \log \text{mobility} = \log \text{cm} \)
PLATE 118

**A**

$R_m = \log (RT - 1)$

PLATE 118

PLATE 123

PLATE 119

**B**

$\log \text{mobility in Cms.}$

PLATE 118

PLATE 123

PLATE 119

HEXOSE UNITS
DISCUSSION

Analytical Techniques

This study gives a more detailed knowledge of oyster hemolymph carbohydrate composition and methods for identification and quantification than any study to date.

The combination of TCA-ETOH fractionation, acid-base hydrolysis and anthrone colorimetric determination worked well to determine broad categories of hemolymph carbohydrate.

The glucose enzymatic determinations also worked well, and were shown to measure glucose if present. However due to the relatively low concentration of glucose present, 4 mg%, it is difficult to obtain good data. Prior concentration of hemolymph by lyophilization might allow either the glucose oxidase or the hexokinase determination to be used with greater accuracy. This may have been the cause of low glucose values in the trehalose assay. Alternatively the HK-NADPH could be sensitive enough, if using better fluorometric equipment a 0.1 mg% sensitivity, as reported elsewhere, could be obtained.

The enzymatic trehalose assay worked very poorly and due to purity of the enzyme, specificity was suspect.
For this reason, and due to the discrepancy between the hemolymph and whole tissue extract results, the quantification of this sugar should be redone. A gas chromatographic study could be done. Due to the apparently low levels of individual FCHO this may be the only method sensitive enough to identify them and permit complete characterization of oyster hemolymph carbohydrates.

A second observation was the amount of FCHO which could not be identified with existing techniques. It was expected that there would be a much higher proportion of glucose, or even trehalose present, being the only "blood sugars" known to date. However there is a discrepancy between the total FCHO value and the sum of the A-B CHO, glucose and trehalose values. Since the chemically stable fraction (A-B CHO) is the largest proportion; consideration was given to the idea that much of the FCHO and A-B FCHO could be maltotriose. TLC showed a relatively large amount present and it was thought that being a trisaccharide it might not hydrolyze as rapidly during the acid-base treatment. Authentic material was treated and it was found that only 5.5% of the initial amount was present by an anthrone determination (Table 11). Assuming that most of the A-B CHO is maltotriose and that the value is 5.5% of the true value, then recalculation gives a value much greater than the total FCHO present. For example, the VIMS May 18 sample examined for A-B CHO was 65 μg/ml, which converts to 1181 μg/ml of maltotriose, this is ten times greater than the FCHO measured independently (Table 4). Repeated attempts were
made to chromatograph the A-B CHO but no method was found to remove the massive amounts of NaOH and other salts from the samples. Therefore, although some of the A-B CHO is probably maltotriose and trehalose, the majority remains unidentified.

The most surprising result of this study was the amount and variability of polysaccharide material present. Unfortunately due to the methods used it is not known if this material is intra- or extracellular; although one would expect it to be intracellular. An interesting study would be to carefully determine where this material is and whether its location is altered under various nutritional, reproductive or environmental conditions. Characterization as to its monosaccharide composition and molecular structure, as well as determination of seasonal alterations should also be done. It seems likely that there is some basal level of PCHO, which is mucopolysaccharide and other structural carbohydrates with a second pool of metabolically active PCHO, probably glycogen.

Composition of Oyster Hemolymph

The total carbohydrate value of 234 to 1420 \( \mu g/ml \) is similar, but slightly higher on the average than those reported for other bivalves or molluscs (Goddard and Martin, 1966; Table 9). The only values previously reported for the oyster specifically was for total reducing substances in Ostrea gigas, 450 \( \mu g/ml \), average, and a range of 300 to 600 \( \mu g/ml \) (Uzuki and Koizumi, 1954; cited by Goddard and Martin,
1966). The presumptive trehalose value found in this study is much lower than that found for whole tissue extracts or other workers (Badman, 1967; Table 9). Whether this is a true difference or reflects methodological problems should be investigated.

The total reducing value in oysters is greater than that found in crustaceans, but, the glucose value for oysters is lower. However, both values are of a similar order of magnitude for the two phyla (Table 9). The TLC indicates the composition of the free carbohydrates is quite similar to the crustaceans. In *Crassostrea virginica* glucose, galactose, maltose/trehalose, and maltotriose were identified. Crustacean free sugars include glucose, glucose-6-phosphate, maltose, maltotriose, fructose, trehalose, and mannose (McWinnie and Sailer, 1960; Meenakshi and Sheer, 1961; Telford 1968, a, b).

In comparison to the insects, according to the traditional view, the total carbohydrate present as well as concentration of glucose and trehalose is quite low in oyster hemolymph (Table 9; Florikin and Jeuniaux, 1964). More recent surveys have indicated that high trehalose hemolymph concentrations may not be as characteristic for the insects as previously thought (Moriarity, 1976; Bedford, 1977). The oyster hemolymph, in winter, does show a similar pattern of total, non-reducing (A-B CHO) and reducing carbohydrate. The non-reducing component is higher than the reducing component and both are higher than can be accounted for by
trehalose or glucose respectively. This suggests that there is a large pool of unidentified reducing and nonreducing carbohydrates whose importance, metabolism and relationship to the other carbohydrate fractions bears investigation.

Glucose is probably the "blood sugar" for the oyster. It is present at levels comparable to those found in crustaceans. In the crustaceans, glucose has been demonstrated to be metabolically active showing variation in response to stress (Telford, 1968a), molt-clycle (Telford, 1968b), and neural-hormonal factors (Quilter, 1977). In the Mollusca, recent evidence has been found for an insulin response and insulin-like proteins, which affect hemolymph glucose levels (Fritsch et al, 1976; De Martines et al, 1973; Marques and Falkmer, 1976).

The low level of glucose is interesting from a nutritional standpoint. Calculations have been made, by Martin (1961) for the metabolic reserve in the hemolymph of oysters based on respiration data showing that under aerobic conditions the oyster's metabolic reserve is 30 minutes. This is high for a sedentary lifestyle considering that similar calculations are: for man, 60 minutes, Helix pomata, 24 minutes, and Octopus dolfeini 28 minutes (Goddard and Martin, 1966). The calculation for oysters is based on 450 μg/ml as the carbohydrate level, not 40 μg/ml for glucose. If glucose is the only sugar used in the hemolymph for intermediate metabolism this would imply a metabolic reserve closer to 3 minutes. This value could be lower, as other respiration values reported are
approximately ten times higher (Galtsoff, 1964; Percy et al 1971). It is likely that hemolymph and tissue storage polysaccharides, and possibly the non-reducing or oligo-saccharides present, are mobilized to maintain hemolymph glucose levels. In the crustaceans there is evidence that the non-glucose reducing sugars are mobilized both seasonally and under stress (Telford, 1968 a, b). Using values found for total APS, the reserve becomes 15 to 90 minutes. However, if the tissue and hemolymph storage carbohydrate levels are maintained at relatively constant levels, with variation being a seasonal phenomenon such a relatively high hemolymph carbohydrate utilization rate, would suggest that constant feeding is necessary to maintain carbohydrate levels. This view of metabolic reserves is consistent with the high feeding rate observed in these animals when active. Also, it is not surprising that total carbohydrate or polysaccharide could drop drastically in response to environmental or reproductive stress.

The role of trehalose is almost certainly not as a storage sugar, since it is present in very low concentrations compared to the other carbohydrate pools in both tissue and hemolymph (Badman, 1967). Two hypotheses have been advanced to explain its presence (Badman, 1967). The first is that it serves to aid glucose absorption by diffusion or facilitated diffusion. This is done by incorporating the transported glucose into another molecule, keeping the glucose concentration gradient high across the gut. This process has been suggested for the locust and crabs where it was found there
is a rise in labelled trehalose or oligosaccharides in the hemolymph after $^{14}$C-glucose is placed in the alimentary canal (Treherne, 1958; Meenakshi and Sheer, 1961). A similar experiment done with a mollusc in vivo and/or in vitro could further our understanding of digestive physiology in these animals.

The second hypothesis is that trehalose and perhaps other oligosaccharides, including maltotriose, are intermediates in various carbohydrate synthetic pathways. The link between trehalose and glycogen synthesis is well characterized in insects, leading to speculation that similar pathways could occur in other invertebrates (Candy and Kilby, 1961; Murphy and Wyatt, 1965; Badman, 1967; Schworch, 1972). Even if similar pathways exist, the metabolic control mechanism would probably be different from the insect system, as in insects a much higher hemolymph trehalose level is maintained in balance with glycogen synthesis. Trehalose, maltose, maltotriose and glycogen synthesis from glucose have been demonstrated in the crustaceans as well as chitin synthesis from glucose, trehalose and maltose (Meenakshi and Scheer, 1961; Schworch, 1972). Glycogen synthesis from a variety of precursors, including trehalose has been demonstrated in the oyster (Fando et al, 1972). In crabs radioglucose appeared in the mono-, oligo-, and polysaccharide fractions successively, suggesting that a glycogen synthesis pathway exists via maltose and maltotriose (Meenakshi and Scheer, 1961). Experiments done in mammalian systems showed similar results, but subsequent work casts doubt on the results (Olavarri, 1960).
Another hypothesis for the existence of the oligosaccharides, particularly the nonreducing ones, would be that they are a source of carbohydrate (glucose) for intracellular polysaccharide synthesis. Use of oligosaccharides might link these synthetic pathways directly to the absorption process or prevent metabolism of the sugars in the hemolymph prior to use for synthesis.

In conclusion, an important fraction of invertebrate hemolymph is the oligosaccharides but their function is unclear.

Geographic and Seasonal Variation

The results of the field survey and seasonal data suggest that hemolymph carbohydrate parameters could be used for studies of oyster condition and nutritional state.

The total APS, FCHO, PCHO, and A-B CHO did not vary greatly with animal size, location or salinity conditions. This was a remarkable result and implies that oyster hemolymph composition is uniform between different populations of oysters. Further, the seasonal variation seemed to follow condition index, (the flesh dry weight as a percentage of shell volume) a parameter already used to characterize the nutritional state of oysters. The individual variation between animals, however, would require a fairly large number of animals being used for each sample. An interesting study would be to measure total anthrone and perhaps glucose or free carbohydrates, for several stations
on various rivers in Virginia for an entire seasonal cycle. Comparison with glycogen content, condition index, gonadal index, temperature, and salinity would provide a better basis for evaluating the stability of hemolymph carbohydrate levels and factors affecting those levels. It is possible that these parameters or another hemolymph parameter could provide a more sensitive indication of nutritional state or natural environmental or pollution stress than other measurements.

The time series examination of the data shows an interesting and unexpected pattern of variation. Using data taken from various sources several parameters were examined to see if on inspection an explanation for the change in carbohydrates could be suggested. These included temperature (N. Windsor, personal communication), phytoplankton (Manzi, 1973), glycogen content (Badman, 1967; Walne, 1970), condition index (Haven, 1960) and gonadal cycle (Kennedy et al, 1964; Loosanoff and Davis, 1952; Loosanoff, 1942). Conclusions are tentative since the data, except for temperature, was obtained from either very different geographical locales or years. This is particularly important as 1977 was an unusually cold winter for Virginia, so seasonal phenomena would be expected to be shifted to later dates. To determine definite correlations, measurements of the environmental variable and hemolymph carbohydrates would have to be done simultaneously and with greater frequency.

Two possible explanations are suggested for the drop and subsequent rise in the hemolymph carbohydrate concentrations observed in this study (Figure 5, A). The first is that this corresponds to mobilization of carbo-
hydrate for gametogenesis.

This seems unlikely, at least for the initial decrease, as it was observed to occur several weeks before the temperature rises to a point where this process can begin. Gonadal ripening began in a Long Island population after the temperature reached 16°C while spawning occurred from the end of June to early July (Loosanoff, 1942; Loosanoff and Davis, 1952). Spawning in Virginia also begins in late June to early July, but may have been later in 1977 (N. Windsor, personal communication). Furthermore, tissue glycogen is rising, not falling, as it does during gametogenesis and spawning (Galtsoff, 1964).

The subsequent rise in hemolymph carbohydrate occurs after the water temperature reaches 16°C, so it is possible this rise reflects mobilization of tissue glycogen into the hemolymph, and then into the gametes in preparation for later development. If true, this would suggest the initial events for gametogenesis would begin two to three months before spawning occurs.

Interestingly the drop in hemolymph carbohydrate occurred after the temperature reached 9°C, the point at which the animals started feeding (personal observation; Galtsoff, 1964). Also the drop and subsequent rise in carbohydrate levels follows a similar pattern to condition index. The latter decreases into March and then rises through May at which point it begins decreasing rapidly due to spawning (Haven, 1960).

Therefore, a second explanation based on nutritional factors is considered. When the oysters begin
pumping, if there is not enough food, one would expect a net decrease in nutritional state. As they use more energy for activity than is assimilated, carbohydrate and perhaps lipid storage pools would be depleted. During this net starvation period one would expect any storage material in the hemolymph to be depleted, but some base level of carbohydrate necessary for cellular metabolism would be maintained, at the expense of both hemolymph and tissue storage carbohydrate. This would reverse when concentrations of the correct food sources increased and the storage pool could be re-established. The drop in total APS as well as the drop in the absolute and proportional concentration of polysaccharide while the proportion of free carbohydrate remains constant is consistent with this idea. This pattern has been observed in experiments with *Carcinus maenas*, both for the hemolymph and tissues. During starvation glycogen levels dropped much more rapidly than glucose (Williams and Lutz, 1975). The drop in condition index and subsequent rise before spawning also supports the idea that the oysters have a net nutritional loss on initially starting to feed, which is compensated for before gametogenesis. The glycogen cycles (Badman, 1967; Walne, 1970) shown do not support this idea, but the data is taken from more southerly populations, therefore the rise and decrease of glycogen may actually occur later and fit better with the hemolymph data.

The available phytoplankton data does not conclusively support or refute the hypothesis that nutritional factors cause the change in hemolymph carbohydrate (Manzi,
<table>
<thead>
<tr>
<th>Species</th>
<th>Total (reducing) CHO</th>
<th>Glucose</th>
<th>Trehalose</th>
<th>Reducing CHO (PCHO)</th>
<th>Non-Reduc. CHO (A-B CHO)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mollusca</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bivalvia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crassostrea virginica</td>
<td>234-1420</td>
<td>40</td>
<td>0.12</td>
<td>87-391</td>
<td>63-242</td>
<td>Present study</td>
</tr>
<tr>
<td>Crassostrea virginica</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Badman, 1967</td>
</tr>
<tr>
<td>Ostrea gigas</td>
<td>300-600</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gastropoda</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strophocheilus oblongus</td>
<td>185-254</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DeJorge et al, 1965</td>
</tr>
<tr>
<td></td>
<td>84-201</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Haeser and DeJorge,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1971</td>
</tr>
<tr>
<td></td>
<td>26-168</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Marques &amp; Falker,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1976</td>
</tr>
<tr>
<td>Australorbis glabratus</td>
<td>20-154</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Becker, 1972</td>
</tr>
<tr>
<td>Lymnaea truncatula</td>
<td>814</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pullin, 1971</td>
</tr>
</tbody>
</table>
TABLE 9 (Continued)
Comparative Hemolymph Carbohydrate Data
μg CHO/ml hemolymph

<table>
<thead>
<tr>
<th>Species</th>
<th>Total (reducing) CHO</th>
<th>Glucose</th>
<th>Trehalose</th>
<th>Reducing CHO (FCHO)</th>
<th>Non-Redu. CHO (A-B CHO)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymnaea stagnalis</td>
<td></td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td>Friedl, 1971</td>
</tr>
<tr>
<td>Cephalapoda</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octopus dolfeini</td>
<td></td>
<td>45-655</td>
<td></td>
<td></td>
<td></td>
<td>Goddard, 1968</td>
</tr>
<tr>
<td>Arthropoda</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crustaceans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orconectes virilis</td>
<td>48-750</td>
<td>10-198</td>
<td></td>
<td></td>
<td></td>
<td>McWinnie &amp; Saller, 1960</td>
</tr>
<tr>
<td>Paranephrops zealandicus</td>
<td>50-300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Quilter, 1977</td>
</tr>
<tr>
<td>Cancer borealis</td>
<td>33-295</td>
<td>4-293</td>
<td></td>
<td></td>
<td></td>
<td>Telford, 1968</td>
</tr>
<tr>
<td>Cancer magister</td>
<td>443</td>
<td>57</td>
<td></td>
<td></td>
<td></td>
<td>Meenakshi and Sheer, 1961</td>
</tr>
<tr>
<td>Homarus americanus</td>
<td>123</td>
<td>79</td>
<td>2.5</td>
<td></td>
<td></td>
<td>Telford, 1968</td>
</tr>
</tbody>
</table>
### TABLE 9 (Continued)
Comparative Hemolymph Carbohydrate Data
μg CHO/ml hemolymph

<table>
<thead>
<tr>
<th>Species</th>
<th>Total (reducing) CHO</th>
<th>Glucose</th>
<th>Trehalose</th>
<th>Reducing CHO (FCHO)</th>
<th>Non-Reduc. CHO (A-B CHO)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insecta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Several orders</td>
<td>50-4x10^4</td>
<td>10-3.2x10^4</td>
<td>2020-5.2x10^4</td>
<td></td>
<td></td>
<td>Florkin &amp; Jeuniaux, 1964</td>
</tr>
<tr>
<td>Earwig (Forrcula auricularia)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Moriaty, 1976</td>
</tr>
<tr>
<td>Centipede (Cormocephalas rubriceps)</td>
<td>234</td>
<td>5.4</td>
<td>trace</td>
<td>95</td>
<td>140</td>
<td>Bedford, 1977</td>
</tr>
<tr>
<td>Dragonfly (Uropetala carovei)</td>
<td>891</td>
<td>60</td>
<td>715</td>
<td>131</td>
<td>759</td>
<td></td>
</tr>
<tr>
<td>Earwig (Anisolabis littorea)</td>
<td>170</td>
<td>48</td>
<td>trace</td>
<td>100</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>
SUMMARY

Techniques have been developed for measurement of various hemolymph carbohydrate fractions by the anthrone method and glucose by specific enzymatic techniques. These hemolymph assays should be useful as a basis for a variety of subsequent investigations of oyster nutrition including seasonal, ecological, nutritional, and biochemical studies. TLC techniques show the carbohydrates present include glucose, galactose, trehalose/maltose, and maltotriose. Trehalose is present in such low concentrations that it certainly does not have a storage role and its metabolic importance is unclear. Glucose is present and in high enough concentration to suggest that it is the "blood sugar". A large portion of the hemolymph carbohydrates remain unidentified and their metabolic role is unknown.
APPENDIX A

Anthrone Reaction

Zubkoff, 1976; personal communication


Hexoses exposed to concentrated sulfuric acid form furfural. The furfural then reacts with anthrone to form a blue-green colored species.

Reagent:
(1) Concentrated sulfuric acid

Anthrone

Thiourea

Add 780 mls of concentrated sulfuric acid to 220 mls of (glass) distilled water in an Erlenmeyer flask. While mixture is warm add 500 mg anthrone and 10 g of thiourea and mix until dissolved. Cool and store in refrigerator. Keeps up to 2 months, but should be checked before use after 2 weeks.

(2) Glucose (or other carbohydrate) standard - 2 mg/ml
Procedure:

The reaction is carried out in 48 x 150 mm test tubes capped with a glass ball (marble) or loose fitting teflon lined cap. An appropriate volume of sample or standard, containing 20 to 200 mg of carbohydrate is added to each tube and the total volume is then adjusted to 1 ml.

Five mls of cold anthrone reagent are added from a burette. The tubes are mixed thoroughly on a vortex mixer and capped. Tubes are placed in a boiling water bath for 15 minutes then they are cooled in a cool water bath for 20 minutes. For uniform color development it is critical that the bath be boiling and that the tubes are cooled uniformly. The color is stable for at least two hours. Absorbance is read at 620 nm on a B & L Spectronic 20 or similar spectrophotometer. A blank and standard must be prepared for each set of samples. Duplication of standards and samples was found to be sufficient. Experimental values can be found either graphically or by calculating them from a linear regression.

Comments:

Small amounts of dirt or lint can cause very erroneous results due to carbohydrate contamination.

For cleanup it is only necessary to rinse tubes two or three times in tap water and once with
distilled water.

**Representative Data:**

A typical set of anthrone data is shown in Table 10. Standard curves for glucose, trehalose and acid-base treated trehalose were done (Figure 13). The anthrone assay was also done on paired sets of 3 samples of glucose, galactose, glucose-1-phosphate, maltose, maltotriose, trehalose, and glycogen. Each sample contained 100 μg of carbohydrate. One set of 3 replicates was first treated with acid and base hydrolysis (Figure 2 C). All acid-base hydrolysis samples were adjusted to 100 μl before adding acid.

The standard curves are, as expected, linear. If one enters an absorbance of 0.01, the average precision to which the scale can be read, this is equivalent to 1.8 μg. Therefore, reported values are reliable to about ± 4 μg. The carbohydrate samples are reported in equivalent μg of glucose. Only trehalose and glycogen have essentially 100% recovery after the acid-base procedure, the other carbohydrates being hydrolyzed. A moderate amount of maltotriose is still present. The value for maltose is higher than the 1-2 μg of equivalent/100 μg reported by Wyatt and Kalf (1957) but is still quite low.
<table>
<thead>
<tr>
<th>Sample</th>
<th>µl CHO solution (2 mg/ml)</th>
<th>µg CHO</th>
<th>ml Dist water</th>
<th>Anthrone Reagent (mls)</th>
<th>Absorbance 1</th>
<th>Absorbance 2</th>
</tr>
</thead>
</table>

**Glucose Standard**

1 0 0 1.00 5 0.000 0.000  
2 10 20 0.990 5 0.074 0.078  
3 20 40 0.980 5 0.155 0.156  
4 50 100 0.950 5 0.40 0.42  
5 100 200 0.900 5 0.77 0.78  

**Trehalose Standard**

1 0 0 1.00 5 0.000 0.000  
2 10 20 0.990 5 0.074 0.074  
3 20 40 0.980 5 0.146 0.150  
4 50 100 0.950 5 0.355 0.375  
5 80 160 0.920 5 0.595 0.61  

**Acid-Base Treated Trehalose**

1 0 0 1.00 5 0.000 0.000  
2 10 20 0.990 5 0.062 0.055  
3 20 40 0.980 5 0.142 0.140  
4 50 100 0.950 5 0.345 0.33  
5 80 160 0.920 5 0.63 0.59
### TABLE 11

**Acid-Base Hydrolysis and Anthrone Determination of Representative CHO's**

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Non-hydrolysed</th>
<th>Hydrolysed</th>
<th>Acid-base as % of non-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Glucose</td>
<td>106</td>
<td>108</td>
<td>112</td>
</tr>
<tr>
<td>Glucose</td>
<td>83</td>
<td>79</td>
<td>79</td>
</tr>
<tr>
<td>Gluc-1-P</td>
<td>18</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Maltose</td>
<td>68</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>113</td>
<td>118</td>
<td>124</td>
</tr>
<tr>
<td>Trehalose</td>
<td>100</td>
<td>100</td>
<td>106</td>
</tr>
<tr>
<td>Glycogen</td>
<td>106</td>
<td>104</td>
<td>101</td>
</tr>
</tbody>
</table>

Six replicate samples, three anthrone determinations done directly, three anthrones done after acid-base hydrolysis. All samples 100 ug of carbohydrate.
APPENDIX B

Hexosamine Reaction

References:
Zubkoff, 1977; personal communication

Procedure is recommended for use with isolated aminosugars since neutral sugars, amino acids and a condensation of neutral sugars and amino acids, can all react with the reagent and give an interfering color.

Reagents:
Acetyl acetone (2, 4 pentanediol)
Redistilled and stored in dark bottle and refrigerate.
0.5 N Sodium carbonate
53.0 grams Na₂CO₃ made to one liter with distilled water.
p-Dimethyaminobenzaldehyde (p-DMAB)
Glucosamine Standard Solution (M.W. 215.60)
1 mg/ml of glucosamine
Reagent A: 20 mls of acetylacteone dissolved in 98.0 mls of 0.5 N sodium carbonate

Reagent B: 542.0 mg of p-DMAB dissolved in 20 mls 1:1 95% ETOH: concentrated HCl, prepared fresh daily

Procedure:

Aliquots of sample containing 0.02 to 0.15 umoles (4.3 to 32.34 µg) of hexosamine or standard up to 50 µgs are placed in screw cap test tubes. The volume is adjusted to 0.6 mls with distilled water. 0.5 ml of Reagent A is added, tubes mixed on vortex and tubes capped loosely with teflon caps or glass marble. Then tubes are heated for 45 minutes in a 90°C water bath. Tubes are cooled in a water bath to approximately room temperature and 2.0 mls of 95% ETOH is added, and tubes are mixed on vortex. After mixing, 0.5 ml of Reagent B are added and tubes vortexed again. A pink to red color should form immediately in the standards. Tubes are left one hour at room temperature and read at 540 nm on a spectrophotometer (B & L Spectronic 20).

Data:

A typical standard curve is given (Table 12). Both hemolymph and the TCA-ETOH extract were used successfully with the procedure. Attempts were also made to use this method with the acid-base hydrolysis material. These gave inconclusive results, sometimes trials worked and in others the proper color failed to develop, leaving all samples with a pale blue
appearance. Probably adjustment of the pH is critical for the reaction. It might be possible by careful adjustment to couple these reactions.
<table>
<thead>
<tr>
<th>Sample</th>
<th>µg of glucosamine</th>
<th>ml of std. solution (1 mg/ml)</th>
<th>ml of Dist Water</th>
<th>Absorbance 1</th>
<th>Absorbance 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.600</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5</td>
<td>0.595</td>
<td>0.075</td>
<td>0.085</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>10</td>
<td>0.590</td>
<td>0.160</td>
<td>0.170</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>20</td>
<td>0.580</td>
<td>0.330</td>
<td>0.328</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>50</td>
<td>0.550</td>
<td>0.78</td>
<td>0.84</td>
</tr>
</tbody>
</table>
ABSORBANCE

0.7 - 0.6 - 0.5 - 0.3 - 0.2 - 0
10 20 30 40 50

µg GLUCOSAMINE
APPENDIX C

Glucose Oxidase Determination of Glucose

Reference:
Sigma Chemical Co. clinical diagnostic kit
Sigma Technical Bulletin 510

Reagents:
Glucose Standard: 1 mg/ml for glucose tests
300 mg% for standard curve
5 mls, Enzyme-color reagent

Procedure:
The instructions in the Sigma Chemical Co.
Bulletin were followed. For each determination a blank
of distilled water, a standard of 100 mg% glucose (0.025 ml
of a 1mg/ml glucose solution) and 0.025 ml of hemolymph.
Often a hemolymph sample with 0.025 ml of glucose was run
to determine recovery efficiency.

For deproteinized samples the semi-micro procedure
was used. To 0.2 ml of distilled water, glucose standard or
hemolymph 1.8 ml of water was added then 1 ml each of Ba(OH)$_2$
and ZnSO$_4$. This was centrifuged 15 minutes at 2600 RPM and
0.5 ml of the clear supernatant used for the determination.
In all tests samples were prepared in individual matched
cuvettes, 5 mls of enzyme-color reagent added and absorbance
read at 450 nm after 45 minutes at room temperature.

**Data:**

A standard curve is shown (Table 13). The determination is linear over the range of 20 to 200 mg% as stated by Sigma. The limit of 20 mg% for accurate measurement is also confirmed, although it might be possible to read half this value, but with uncertainty.
### TABLE 13

**Glucose Oxidase Standard Curve**

<table>
<thead>
<tr>
<th>Sample</th>
<th>mg% glucose</th>
<th>µl 300mg% glucose stnd soln</th>
<th>Dist. water to obtain proper mg% mls</th>
<th>Dist water for Rx volume mls</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>0.525</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>0.040</td>
<td>0.460</td>
<td>0.525</td>
<td>0.054</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0.085</td>
<td>0.415</td>
<td>0.525</td>
<td>0.125</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>0.165</td>
<td>0.335</td>
<td>0.525</td>
<td>0.255</td>
</tr>
<tr>
<td>5</td>
<td>300</td>
<td>0.50</td>
<td>0.0</td>
<td>0.525</td>
<td>0.76</td>
</tr>
</tbody>
</table>

All samples incubated 45 minutes at room temperature with 5 ml enzyme reagent prepared according to Sigma Bulletin 510
APPENDIX D

Hexokinase-NADPH Fluorometric Glucose Determination

Reference:


Reagents:

Sigma Glucose Diagnostic Reagent (Bulletin No. 15-UV)

1.3 total volume of reconstituted enzyme reagent per vial

Glucose standard 5, 10, 50 mg°/oo

Diluted reagent:

0.01N NaOH

1 mM Sodium EDTA

0.3 N Ba(OH)₂

5% ZnSO₄·7H₂O

Procedure:

10 μl of distilled water (blank), glucose solution (standard) and hemolymph are added to 20 μl of BaOH in a
1.5 ml conical dispo-micro beaker (Scientific Products, McGraw Park, Ill). Twenty μl of ZnSO₄ are added, the solution mixed and centrifuged for 10 minutes at 2600 RPM. Ten μl of each supernatant are transferred to 1 ml micro test tubes. Two hundred μl of enzyme reagent are added and the reaction allowed to occur for 15 minutes at room temperature. Then, 300 μl of dilutant are added to stop the reaction. Readings are taken on a fluorometer (Turner Model 111) with an excitation wave length of 360 and fluorescence wave length of 465 nm. The Turner 111 fluorometer was equipped with a high sensitivity door, a primary filter of 7-54 (#110-810 - passing all wave lengths 254-420 nm) and a secondary filter No.3 (#110-827 passing all wave lengths above 455 nm). The scale factor is set empirically depending on the samples. However it was found necessary to re-zero with the blank for each scale. Samples should be read immediately as there does seem to be a slow destruction of the NADPH.

Considerable problems were encountered, at first, in obtaining consistent readings. It was found that the micro cuvettes were of sufficiently poor optical quality that even a small reorientation in the fluorometer could cause a large change in the reading. Therefore, a single cuvette was placed in the machine and the samples pipeted with separate Pasteur pipets. Also since a slight drift occurred with the higher scale factors three successive readings were taken by zeroing the fluorometer, taking a set of readings for all samples, rezeroing and taking a second set of readings. Using this method standard
deviation for 5 replicas of 10 mg% was 0.93 mg% (Table 14). This is not as good as reported by previous workers, (Quilter, 1977) having a S.D. of 0.43 mg% for 20 trials of 10 mg% glucose samples.

A standard curve was done (Table 14; Figure 16). It is linear at the lower concentration and shows the slight non-linearity previously reported; but this results in only a small error (Quilter, 1977; Scherstein and Tibbling, 1967).
TABLE 14

Hexokinase-NADPH Glucose Determination-Standard Curve

<table>
<thead>
<tr>
<th>Sample</th>
<th>mg% glucose</th>
<th>Scale factor</th>
<th>Fluorometer Reading (Arbit. Units)</th>
<th>Adjusted Fluorometer Readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>1x</td>
<td>off scale</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>1x</td>
<td>60.5 63</td>
<td>1815 1890</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>1x</td>
<td>39.5 39.5</td>
<td>1155 1185</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>1x</td>
<td>20.5 19</td>
<td>615 570</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>10x</td>
<td>9.5 12</td>
<td>285 360</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>30x</td>
<td>64.5 58</td>
<td>64.5 58</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>30x</td>
<td>18 17.5 22</td>
<td>18 17.5 22</td>
</tr>
<tr>
<td>8</td>
<td>0.5</td>
<td>30x</td>
<td>12 17 16</td>
<td>12 17 16</td>
</tr>
<tr>
<td>9</td>
<td>0.0</td>
<td>1,10,30x</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

Replication

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorometer Readings</th>
<th>Fluorometer Readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28 23 26</td>
<td>Average 25.2</td>
</tr>
<tr>
<td>2</td>
<td>23 25.5 18</td>
<td>S.D. 2.315</td>
</tr>
<tr>
<td>3</td>
<td>26 18 17</td>
<td>S.D. in mg% 0.93</td>
</tr>
<tr>
<td>4</td>
<td>27 14 14</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>22 16 14</td>
<td></td>
</tr>
</tbody>
</table>

Five 10 mg% glucose sample. Reaction and readings taken according to methods and materials, Appendix D.
APPENDIX E

Trehalose - Trehalase - HK - NADPH Coupled Assay

Reference:


Reagents:

Glucose standard 50 mg%
Trehalose standard 30 umoles/30μl
Cacodylate buffer PH 6.5 20.0 umoles/50μl
Trehalase
Sigma glucose - Hexokinase enzyme
Dilutant solution (0.01 m NaOH, 5 mm NaEDTA)

Procedure:

For each determination a blank, glucose, standard, trehalose standard, hemolymph without trehalase, hemolymph with trehalase, hemolymph with trehalase and spiked with trehalose were run in micro centrifuge tubes (see Table 15 for reaction mixture). The enzyme was the final addition and the samples were incubated one hour in a 37°C water bath. The reaction was stopped by placing them in boiling water 10 minutes.
The tubes were cooled and 200 µl of the hexokinase enzyme reagent added. After 15 minutes at room temperature 300 µl dilutant solution was added and reading taken on the fluorometer. Readings were taken on the 10x scale with a 10% neutral density filter. The use of the filter seemed to stabilize the reading somewhat. The trehalase plus hemolymph sample minus the hemolymph sample alone (correction for natural glucose) is presumably the glucose due to authentic trehalose. The hemolymph spiked with trehalose minus the hemolymph with trehalase allows computation of the percent recovery of trehalose.
### TABLE 15

Trehalose Assay Reaction Mixture

<table>
<thead>
<tr>
<th>Sample</th>
<th>µl of Reagent Solution</th>
<th>Dist.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer¹</td>
<td>Trehalase</td>
</tr>
<tr>
<td>1 Blank</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>2 Glucose Stnd.</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>3 Trehalose Stnd.</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>4 Hemolymph Glucose</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>5 Hemolymph Trehalose</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>6 Hemolymph + Trehalose Spike</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>7 Maltose</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>

¹ Cacodylate buffer, pH 6.5, 20 µmoles/50µl
² 10 mg% or 1 µg/10µl
³ 3 µmoles/30µl

Hemolymph glucose = 4

Hemolymph trehalose = 5 - 4 = glucose from hemolymph trehalose + glucose - hemolymph glucose

% recovery trehalose = (6 - 5)/3 x 100%

= glucose from trehalose spike in hemol.

= glucose from equivalent amt. trehalose
APPENDIX F

Thin Layer Chromatography Visualization Methods

Methods used with crustaceans (Telford, 1965, 1968; McWinnie and Saller, 1960), insects (Moriarity, 1976; Doctor and Sale, 1976; Wyatt and Kalf, 1957) and other invertebrates (Badman, 1967; Fairbain, 1958) were examined, particularly relative to trehalose visualization. General works on chromatography were consulted as well (Stahl, 1969; Dawson et al, 1959; Hough, 1954). One specific method for trehalose visualization was found, but could not be tried in this study (Caldes and Prescott, 1975).

Ammoniacal silver nitrate (Stahl, 1969)
Aniline malonate (Caldes and Prescott, 1973)
Ansidine (Dawson et al, 1959)
Benzidine (Dawson et al, 1959)
Diphenylamine-aniline-phosphoric acid (Stahl, 1969)
Phthalic acid (Dawson et al, 1959)
Naphthoresorcinol (Stahl, 1969)

In order to screen a variety of reagents silica gel and cellulose plates were prepared with pairs of spots, the first with glucose, trehalose and glucose-1-phosphate, the second with lactose and maltose. The silica gel plates were developed in n-butanol:ETOH:water 4:1:1.9, and the
cellulose plates in ethyl acetate:pyridine:water:glacial acetic acid:propanoic acid 50:50:10:5:5. After development and drying the plates were cut into strips, each strip containing a pair of standards. Each strip was developed with a different reagent. A summary results appears in Table 16.

Most reagents visualized the reducing saccharides, but a few did not visualize maltose particularly well. The most sensitive reagents were diphenlyamine, naphthoresorcinol and silver nitrate. The limits of detection for naphthoresorcinol were tested by spotting dilutions of glucose standard (2 mg/ml) on silica gel and developing with n-butanol:ETOH:water. It easily visualized glucose trehalose and glucose-1-phosphate to the 1 μg/spot but the limit of detection is probably between 0.1 and 0.5 μg/spot. The limit for maltose and glactose was 5 μg.

The AgNO₃-NaOH method had two problems. First although it was quite sensitive in detecting most sugars the background color was very dark on both the cellulose and silica gel plates. It also failed to detect trehalose consistently, although on heating (105°C 15-30 seconds) a 10 μg spot could be visualized, but results were not constant. Therefore the method of identifying trehalose described by Badman (1967) could not be used. Considerable problems were also encountered using the cellulose plates. The napthoresorcinol reagent, with 10% tricloracetic acid, gave an even pink color with no visualization of carbohydrates. The AgNO₃-NaOH gave a high background color due to the carbohydrate
binder Merck uses to "harden" the plates. Of the reagents used naphthoresorcinol, diphenylamine and benzidine seemed to be most sensitive and gave reproducible results, therefore they were used throughout this study. The benzidine naphthoresorcinol combination with each sprayed on replicate plates seemed the best combination for differentiating trehalose and maltose by chemical means.
### TABLE 16
Thin-Layer Chromatography Visualization Reagents

<table>
<thead>
<tr>
<th>Reagent/Treatment</th>
<th>Glucose</th>
<th>Glucose Trehalose</th>
<th>Maltose</th>
<th>Maltotriose</th>
<th>Gluc-1-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica Gel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silver nitrate-sodium hydrox.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Room Temperature</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>105°C 15 sec.</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Anisidine</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Aniline malonate</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aniline phthalate</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Benzidine</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Peridate+phthalate</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Diphenylamine, aniline, phosphoric acid</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Napthoresorcinol</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Peridate-AgNO₃-NaOH</td>
<td>++++</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 16 (Continued)

**Thin-Layer Chromatography Visualization Reagents**

<table>
<thead>
<tr>
<th>Reagent/Treatment</th>
<th>Glucose</th>
<th>Glucose</th>
<th>Trehalose</th>
<th>Maltose</th>
<th>Maltotriose</th>
<th>Gluc-1-P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellulose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AgNO₃-NaOH + Heat</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>not done</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Anisidine</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>Benzidine</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>Periodate+benzidine</td>
<td>++⁺⁺⁺</td>
<td>+⁺⁺⁺⁺</td>
<td></td>
<td></td>
<td></td>
<td>&quot;</td>
</tr>
</tbody>
</table>

119
BIBLIOGRAPHY


VITA

Robert Joel Lowy

Born in Pittsburgh, Pennsylvania, August 24, 1952. He graduated from Penn Hills High School in 1970 and from College of William and Mary in 1974 with a B.S. in Biology. He entered the School of Marine Science in September 1974. He will begin a doctoral program in the Department of Zoology at Oregon State University in September, 1977.